Hypoxia and the Neuronal Cytoskeleton

A Role for cdk5/p35

DISSERTATION

Zur

Erlangung der naturwissenschaftlichen Doctorwürde

(Dr.sc.nat.)

Vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

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Zürich, 2008

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1 Abbreviations

AMPA	Alpha-amino-3-5-methyl-4-isoxazolproprionic
AMS	Amyotrophic lateral sclerosis
ARNT	Aryl hydrocarbon receptor nuclear translocator
Aβ	Beta-amyloid
ATP	Adenosine tri-phosphate
CNS	Central nervous system
CSF	Cerebrospinal fluid
Cdk5	cyclin dependent kinase 5
DIV	Days *in vitro*
EMSA	Electrophoretic mobility shift assay
Epo	Erythropoietin
GSK3	Glycogen synthase kinase 3
GLUT1	Glucose transporter 1
Hif-1	Hypoxia-inducible factor-1
Hx	Hypoxia
HRE	Hypoxia responsive element
JNK3	Jun N-terminal kinase 3
LDH	Lactate dehydrogenase
MAGE-D1	Melanoma associated antigen-D1
MEF2	Myocyte enhancer factor 2
NMDA	N-methyl-D-aspartic acid
NFs	Neurofilaments
NFTs	Neurofibrillary tangles
Nx	Normoxia
RT-PCR	Real-Time PCR
VHL	Von Hippel-Lindau
PHD	Prolyl hydroxylase
VEGF	Vascular endothelial growth factor

2 Summary

Constant oxygen and glucose supply is required to support structural and functional integrity of the brain. Hypoxia is a major stress stimulus for neurons, however susceptibility to hypoxia varies considerably depending on the severity of the stimulus, the affected area and the type and age of cell. While hypoxia is known to affect the cytoskeleton, recent evidence further suggests that cytoskeletal alterations could also be involved in regulation of Hif-1, the main modulator of oxygen-dependent gene expression. At the same time evidence suggest a role for the cdk5/p35 complex, an important regulator of the cytoskeleton, in the neuronal response to hypoxia. Cdk5 is a serine/threonine kinase that controls many different pathways through phosphorylation of a wide array of proteins. In turn, cdk5 activity is regulated by association with its neuronal specific activators p35 and p39 and their cleaved products p25 and p29 respectively. The cdk5/p35 complex is essential for cell survival in development. At the same time, deregulation of the cdk5/p35 complex contributes to the pathogenesis of neurodegenerative disorders.

The main focus of this study was to elucidate the early events that contribute to neuronal death following hypoxia and to identify variations of responses at different neuronal ages. In particular, we were interested in the contribution of the cytoskeleton and cytoskeletal-regulatory proteins in the neuronal hypoxic response.

Our data show that hypoxia leads to a 35% increase in cell death in mature cultures as opposed to only a 10% increase observed in immature neurons suggesting that mature neurons are more susceptible to hypoxic injury. Concomitantly, hypoxia significantly disrupted the neuronal F-actin cytoskeleton demonstrating the response of the neuronal cytoskeleton to oxidative stress. Furthermore we provided evidence for a

role for the cdk5/p35 complex in the neuronal response. In both immature and mature neurons hypoxia transiently increased cdk5 and p35 mRNA levels and cdk5 activity. Age-dependent regulation of the cdk5/p35 complex by hypoxia was also observed with cleavage of p35 to p25 only in mature cells. Application of roscovitine, a pharmacological inhibitor of cdk5, exacerbated hypoxia-induced F-actin disruption suggesting a role for cdk5 in maintenance of the hypoxic cytoskeleton in both ages.

In all neurons, increased activation of cdk5 correlated with elevated Hif-1α expression and binding activity, whereas cdk5 inhibition downregulated Hif-1α stabilization and also affected Hif-1α target gene expression. The application of roscovitine during extended hypoxia significantly induced death in young neurons, suggesting a specific role for cdk5 in modulation of neuronal survival pathways solely in long-term injury/insult.

This work also aimed to study whether the astrocytic response to hypoxia differs from that of neurons. Astrocytes are able to maintain ATP levels and thus survive oxygen deprivation for long periods of time, as opposed to neurons which respond to hypoxia with a dramatic drop in ATP and an earlier onset of cell death. Importantly, unlike the immediate Hif-1α stabilisation in neurons following hypoxia, Hif-1α in astrocytes is stabilized only in near anoxic conditions, further indicating the resistance of astrocytes to oxygen deprivation.

Taken together this work provides evidence of specific regulation of important pathways not only between different brain cell types but also age-dependent regulation. Notably this work provides evidence for the interaction of two important pathways, the Hif hypoxic pathway and cdk5 an important player in neurodegeneration.

Zusammenfassung

Um die strukturelle und funktionelle Integrität des Gehirns aufrechtzuerhalten, wird eine konstante Sauerstoff- und Glukosezufuhr benötigt. Innerhalb des zentralen Nervensystems variiert die Anfälligkeit gegenüber Hypoxie (Sauerstoffmangel), abhängig von der Stärke des Stimulus, der betroffenen Region, des Zelltyps und deren Alter. Hypoxie ist ein bedeutender Stressfaktor für Neuronen und beeinträchtigt unter anderem das Zytoskelett. Jüngste Forschungsergebnisse deuten daraufhin, dass Änderungen im Zytoskelett an der Regulation von Hif-1, dem wichtigsten Transkriptionsfaktor sauerstoffabhängiger Gene, beteiligt sind. Cdk5 ist eine Serin/Threonin-Kinase die mittels Phosphorylierung verschiedenster Proteine eine Vielzahl von Signalkaskaden kontrolliert. Die Aktivität von cdk5 wiederum wird von seiner Komplexbildung mit den neuronal spezifischen Aktivatoren p35 und p39 und deren Spaltprodukten p25 respektive p29 reguliert. Der cdk5/p35-Komplex ist notwendig für die Regulation des Zytoskeletts und der Überlebensfähigkeit der Zellen während der Entwicklung. Gleichzeitig tritt die Deregulierung des cdk5/p35-Komplexes in verschiedenen neurodegenerativen Krankheiten auf.

Ziel dieser Studie war, die frühen Vorgänge zu identifizieren, die zu neuronalem Zelltod als Folge von Sauerstoffmangel führen, sowie Variationen in der Reaktion von Neuronen unterschiedlichen Alters aufzuzeigen. Im Besonderen waren wir an der Rolle des Zytoskeletts und dessen regulatorischen Proteinen in der neuronalen Reaktion auf Hypoxie interessiert.

Unsere Ergebnisse zeigen, dass Hypoxie in reifen neuronalen Zellkulturen den Zelltod um 35% erhöhte, in unreifen Neuronen hingegen betrug die Zunahme nur 10%. Somit scheinen reife Neuronen anfälliger für hypoxische Schäden zu sein. Gleichzeitig beschädigte Hypoxie signifikant das neuronale Zytoskelett von F-Aktin und

bestätigte so den Einfluss von oxidativem Stress auf das Zytoskelett der Neuronen. Zusätzlich scheint der ckd5/p35-Komplex eine Rolle in der neuronalen Reaktion auf Hypoxie zu spielen. Sowohl in unreifen wie auch in reifen Neuronen führts Hypoxie zu vorübergehend erhöhter ckd5 und p35 mRNA Expression sowie gesteigerter cdk5 Aktivität. Wir beobachteten auch eine altersabhängige Regulation des cdk5/p35-Komplexes durch Hypoxie; das Spaltprodukt von p35, p25, allerdings trat nur in reifen Neuronen auf. Die Anwendung von Roscovitine, einem pharmakologischen cdk5-Hemmer, verstärkte die durch Hypoxie verursachten Schäden an F-Aktin, was darauf hindeutet, dass cdk5 zur Aufrechterhaltung des hypoxischen Zytoskeletts beiträgt, unabhängig vom Alter der Zellen.

In allen Neuronen korrelierte die gesteigerte Aktivität von cdk5 mit erhöhter Hif-1α Expression und Bindungsaktivität. Die Hemmung von cdk5 hingegen verminderte wesentlich die Stabilisierung von Hif-1α und beeinträchtigte ebenso die Expression der Hif-1α-regulierten Gene. Wir folgern daraus, dass die cdk5-Signalkaskaden an der Aktivierung der Reaktion auf Hypoxie beteiligt sind. Die Anwendung von Roscovitine über längeren Perioden von Hypoxie führte zum Zelltod junger Neuronen, was auf eine spezifische Rolle von cdk5 in der Regulierung von neuronalen Überlebenssignalwegen während Langzeitstress hinweist.

Ziel dieser Studie war des Weiteren, zu untersuchen, ob sich die Reaktion von Astrozyten auf Hypoxie von derjenigen von Neuronen unterscheidet. Astrozyten konnten ihre ATP-Werte beibehalten und überlebten somit längere Zeit ohne Sauerstoffzufuhr, während hingegen in Neuronen die ATP-Werte dramatisch zurückgingen und der Zelltod eintraf. Bemerkenswerterweise führte Hypoxie nur in Kombination mit Glukosemangel bei Astrozyten zum Zelltod, während sie indes alleine keinen signifikanten Einfluss auf das Überleben der Zellen hatte. Somit

stabilisieren Astrozyten Hif-1α nur unter nahezu anoxischen Bedingungen kombiniert mit erhöhtem Zelltod, Neuronen andererseits reagieren mit sofortiger Hif-1α Stabilisierung auf Sauerstoffmangel. Kurz, Astrozyten zeigen sich resistent gegenüber Sauerstoffmangel.

Zusammenfassend zeigt diese Studie Beispiele von spezifischer Regulation wichtiger Signalkaskaden in Funktion des Typs sowie des Alters der involvierten Zellen. Des Weiteren sprechen unsere Ergebnisse für eine Verbindung zwischen der Hif-Hypoxie-Signalkaskade und cdk5, einem Schlüsselfaktor in neurodegenerativen Prozessen.

4 INTRODUCTION

HYPOXIA

Oxygen (O_2) is essential for life. In mammals, fine-tuned respiratory and cardiovascular systems have evolved and contribute to appropriate cellular and tissue distribution of O_2. In turn O_2 serves as the terminal electron acceptor during mitochondrial oxidative phosphorylation, the main biochemical reaction for generating energy in the form of ATP. Insufficient oxygen supply results to a switch from aerobic to anaerobic metabolism, which can be employed by organisms for short periods of time. Nevertheless the tolerance to changes in O_2 levels varies considerably depending on the species (2).

Hypoxia is defined as a condition in which oxygen availability/delivery to the tissue is below physiological levels (3). Reduced oxygen availability (hypoxia) occurs in many physiological situations including embryonic development, adaptation to high altitude, as well as pathophysiology of cancer, stroke, and neurodegenerative disorders, such as Alzheimer's disease (4, 5, 6, 7). Higher organisms have recruited a number of adaptive mechanisms in order to ensure efficient supply of oxygen upon demand. A number of studies on mountain climbers have reported acclimatization to high altitude and thus hypoxia, by a number of processes that include: i) progressive increase in ventilation, ii) adaptations in the cardiovascular system (i.e. increase of vascular density in heart and brain) that ensure enhanced oxygen delivery to tissues, and iii) alterations at the cellular level for a more efficient utilization of available oxygen (8),(9),(10). However, a lot is still to be revealed about the molecular pathways that are involved in the organism's response to low oxygen levels. Understanding the impact of hypoxia is both physiologically relevant to elucidate the

adaptive mechanisms in response to high altitude, as well as clinically relevant towards our understanding of the pathophysiology of many diseases.

4.1.1 Hif-1 as a mediator of the hypoxic response

The identification of the transcription factor, hypoxia-inducible factor-1 (Hif-1) in 1995 by Wang and Semenza (11) was a hallmark in our understanding of oxygen physiology. Nowadays it is widely accepted that at the molecular level, sensing and responding to hypoxia involves the stabilisation of Hif-1. The importance of Hif-1 in regulating hypoxic responses is further underlined by the fact that it is highly conserved throughout evolution ranging from Drosophila to humans (12).

The Hif complex is a heterodimer composed of a constitutively expressed Hif-β subunit (also known as aryl hydrocarbon receptor nuclear translocation, ARNT) and a Hif-α oxygen regulated subunit (13-16). Both Hif-α and Hif-β exist as isoforms (Hif-1α, Hif-2α, Hif-3α and ARNT, ARNT2 and ARNT3 respectively). Regulation of Hif-α activity occurs at different levels that include mRNA expression, protein stability, nuclear translocation and activity, all being influenced by alterations in oxygen levels. At the molecular level, Hif-α protein stability is largely regulated by hydroxylation of proline residues within the Hif-α sequence. It is now known that hydroxylation involves a family of prolyl hydroxylases, namely PHD1, PHD2, and PHD3 (reviewed (17-21). Hydroxylation promotes the binding of von Hippel-Lindau (VHL) tumour suppressor protein to Hif-α targeting the latter to proteosomal degradation. However, upon hypoxia PHD activity decreases thus allowing the rapid accumulation of Hif-α. Stabilised Hif-1α translocates in the nucleus and dimerises with Hif-β ultimately enhancing transcriptional activity of its target genes by binding to the hypoxia response element (HRE). A number of other proteins have been shown

to contribute to Hifα stability and activation, a few examples of which are provided in Table 1.

Hif-1 interacting Proteins	**Protein modification and stability**
PHD1/PHD2/PHD3	**Prolyl hydroxylation**
VHL	**Ubiquitination**
FIH	**Asparaginyl hydroxylation**
HSP90/HSP70	**Folding/stability**
P53	**Protein stability**
Mdm2	**Protein stability**
SUMO-1	**Protein stability/transcriptional activity**
P42/p44ERK	**Phosphorylation**
GSK3	**Phosphorylation**

Table 1. Hif-1 interacting proteins involved in regulation of Hif-1 stabilization and/or activity. Regulation is achieved by modifications such as hydroxylation, ubiquitination, acetylation, and/or phosphorylation.

It is well established that activation of the Hif transcription system is the result of alterations in oxygen levels, and serves as an adaptation mechanism to maintain oxygen homeostasis. To date, more than 100 Hif-1 target genes have been identified, and the list is expanding. As shown in Table 2, the functions of many of these genes are described in the literature and are involved in important processes such as oxygen supply, cellular metabolism, and cell growth and apoptosis (22). Amongst others, the vascular endothelial growth factor (VEGF), erythropoietin (Epo) and glucose-transporter 1 (GLUT1) are well characterised Hif-target genes and of particular interest in this study, since they are involved in proliferation, cell survival, and energy metabolism.

Hif regulated Gene	Function	Reference
Increased oxygen supply		
Erythropoietin (Epo)	Erythropoiesis	(23)
Endothelin	Vessel diameter	(24)
Globin-2	Oxygen transport	(25)
Flt-1/VEGF-R1	Angiogenesis	(26)
Flk-1/VEGF-R2	Angiogenesis	(27)
eNOS	Vessel diameter	(28)
Heme-oxygenase	Vessel diameter	(29)
VEGF	Angiogenesis	(26)
Cellular Metabolism		
Aldolase	Glycolysis	(30)
GLUT1	Glucose uptake	(31)
GLUT3	Glucose uptake	(32)
Lactate dehydrogenase	Glycolysis	(30)
Cell Growth and Apoptosis		
BNIP3	Pro-apoptotic	(33)
NIP-3	Pro-apoptotic	(34)
TGF-β3	Placenta development	(35)

Table 2. Selected Hif-1 target genes.

Numerous studies have provided evidence for the fundamental role of Hif both in development but also in the adult system. Embryonic lethality at gestational day 10 (E10) with severe defects in blood vessel and heart development has been reported in

mice lacking the Hif-1 functional gene (30, 31). Several additional studies have confirmed the pro-survival role of Hif in chronic hypoxic conditions. However under certain circumstances Hif-1 can also act as a pro-death signal. The first evidence came from studies on cancer cells and the pro-apoptotic tumour suppressor p53 (36) that showed that hypoxia in tumours induces Hif-1α, which in turn stabilises p53 thereby leading to apoptotic death. Since then similar mechanisms have been described to be employed by neurons in models of ischemic injury (37).

To date scientists agree that Hif-1 function as a pro-survival or pro-death signal is dependent on the type of cell, the degree and duration of hypoxia, as well as the relative levels of Hif-1 present in the cell (Fig.1).

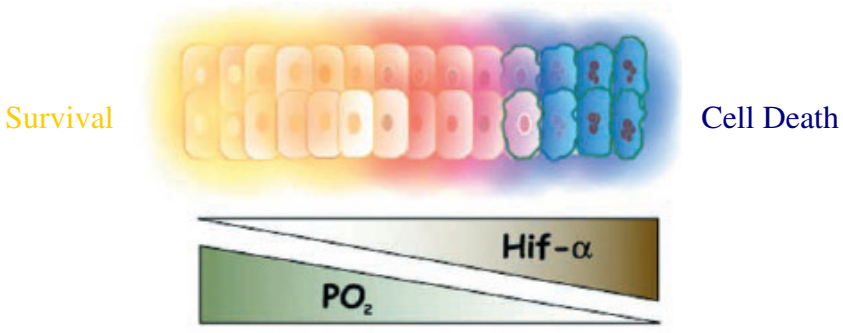


Fig. 1. Partial oxygen pressure (PO_2) and Hif-1α are crucial factors in determining the role of Hif in increasing cell survival or inducing cell death. In response to a decrease in oxygen availability, cells react with Hif-1α induction which can function as a pro-survival or pro-death signal depending on the degree and duration of hypoxia but also on the levels of Hif-1 present in the cell (Figure by (18))

THE BRAIN.

"From the brain and the brain alone arise our pleasures, joys, laughter and jests, as well as our sorrows, pains and grief" *Hippocrates*

The human brain constitutes only 2% percent of our body weight, yet, it has been estimated that proper brain function involves energy consuming processes that account for approximately 25% of total body glucose utilization (38). The three major cell types of the brain are: 1) neurons, 2) astrocytes and 3) oligodendrocytes. A brief introduction on neurons and astrocytes, the cell types under investigation in this study will follow.

4.2.1 The neuron

The neuron is the basic working unit of the brain. This highly specialised cell is composed of a cell body (soma) containing the nucleus and cytoplasm and the axon, an electrically excitable output fiber (Fig.2) as well as dendrites that extend from the neuronal soma, and can be distinguished from axons by their size.

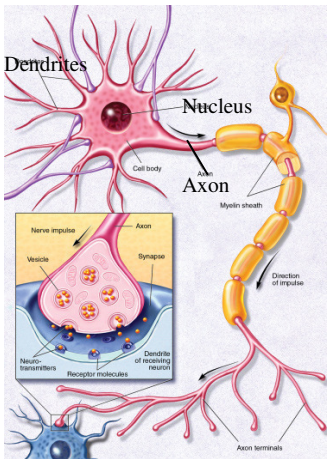


Fig.2. Schematic representation of a neuron composed of a neuronal soma, the axon and several dendrites. Electrical signals travel along the axon and when reaching the synapse they trigger the release of neurotransmitters. The latter bind to receptors on the surfaces of adjacent neurons, hence ensuring neuronal communication. (Figure taken from www.sfn.org: Brain Facts).

Neuronal signals are transmitted in the form of a nerve impulse along the axon, and involve the opening and closing of ion channels. ATP-driven ion pumps, involved in reverse ion fluxes associated with action potentials and excitatory postsynaptic currents represent a major energy drain for the brain. Information is subsequently transmitted from one cell to the other by release of substances that are known as neurotransmitters. 10-70% of the total energy consumption in the brain is required to support neurotransmission (39). Maintenance of functional connections and regulation of neural activity is essential throughout life to ascertain normal brain functioning. Prolonged periods of increased neuronal activity are linked to brain dysfunction and can often lead to neuronal death. It thus follows that disturbances in oxygen levels and thus in energy metabolism can have deleterious effects in brain function.

4.2.2 The astrocyte

Astrocytes are characteristic star shaped cells with their many processes spanning around neurons (Fig.3). During development, astrocytes derive from radial glial cells which serve as scaffolds for neuronal migration thus contributing to defining the CNS cytoarchitecture. As the CNS matures, radial glial cells serve as progenitors of astrocytes which in adulthood enseath neurons and vessels of the CNS (40). The ratio of astrocytes to neurons increases dramatically with brain complexity, ranging from a 1:3 ratio in the cortex of lower mammals, to 10:1 in the human cortex (41). It is suggested that the evolution of sophisticated neuronal networks required greater degree of local control, which in the human brain involves the astrocytes. Thus, although astrocytes were initially thought to be only involved in structuring the brain they are now considered to play an active role. To date there is strong evidence for the role of astrocytes in modulating neuronal function. A study by Pfrieger et al., (42), reported that neurons co-cultured with astrocytes develop more synapses compared with neurons growing in the absence of asrocytes. Subsequent studies revealed that in the adult rat hippocampus astrocytes might be involved in neurogenesis (43). Additionally astrocytes were shown to be involved in modulation of neuronal activity. Following neural activity-induced increases in extracellular K^+ levels, astrocytes contribute to K^+ homeostasis by either redistributing or transiently sequestering K^+ (44). In a similar manner, astrocytes participate in glutamate synthesis and removal, thus indicating an important role for these cells in the modulation of excitatory synapses (45).

Notably, functions of astrocytes are not restricted to neuronal regulation. It is widely recognised that astrocytes contribute to the formation of the blood-brain barrier via

induction of endothelial tight junction formation (46). This is made possible by the close interaction of endothelial cells with the astrocytic end feet processes. The position of astrocytes in the brain, and their close contacts with both endothelial and neuronal cells (Fig.3), have prompted scientists to postulate that these cells act as messengers between neurons and vascular cells during normal brain function and most importantly in cases of increased energy demand.

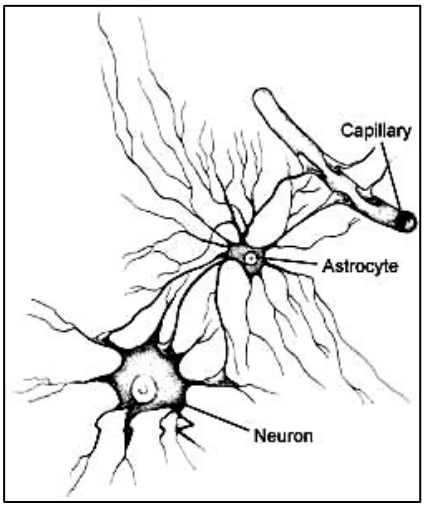


Fig.3 Schematic representation of an astrocyte, and its contacts with both neurons and the capillaries. The astrocytic end feet processes of the astrocytes make contacts with both neurons but also endothelial cells in the brain (Figure taken from www.aboutmind.com).

4.3 BRAIN AND DISEASE

Understanding how neurons are born, develop and organise themselves into functional and effective systems, is a major challenge for neuroscientists but is essential for understanding how the brain responds to external influences or injury. Impairment of neuronal function leads to various neurological disorders. Mortality as a result of neurological disorders constitutes 12% of total death globally (Table 3), while nervous system diseases lead to more hospitalisations that any other disease

group including heart disease and cancer, urging researchers to find ways for preventing or curing many devastating brain disorders (www.sfn.org).

Hypoxia is implicated in the pathogenesis of many central nervous system disorders. Hypoxia and ischemia are common forms of injury during the perinatal period (47). Injury due to hypoxia can occur in premature infants as well as infants at term as a result of pulmonary insufficiency, or birth asphyxia respectively (48). At the same time alterations in oxygen delivery to the tissue characterise head trauma, stroke, brain tumours, and a number of neurodegenenarative disorders such as Alzheimer's disease.

Cause category	2005 (%)
Epilepsy	0.22
Alzheimer and other dementias	0.73
Parkinson's disease	0.18
Multiple sclerosis	0.03
Migraine	0.00
Cerebrovascular disease	9.90
Poliomyelitis	0.00
Tetanus	0.33
Meningitis	0.26
Japanese encephalitis	0.02
Total	**11.67**

Table 3. Common diseases of the CNS and % of death attributed to them globally. (Table from: www.who.int/entity/mental_health/neurology/neurological_disorders_report_web.pdf).

4.3.1 The hypoxic brain

Functional integrity of the brain depends on constant oxygen and glucose supply. To combat metabolic changes and toxicity and to ensure maintenance of oxygen homeostasis, several short-term and long-term adaptive mechanisms have evolved. Short-term responses include: i) cerebral autoregulation, a process which describes a local increase in blood flow as a result of regional imbalances in oxygen levels, and thus of increased neuronal activity, and ii) reversible inhibition of translation resulting in shutting down non-vital energy consuming mechanisms (2).

Concomitantly, hypoxia leads to the activation of the Hif system, which also in the brain is responsible for the activation of genes that reduce oxygen consumption and increase oxygen delivery to the injured region. In the adult rat, basal Hif-1α and Hif-1β mRNA expression is detected in most brain regions (49-51), whereas low levels of Hif-1α protein expression is mainly present in hippocampal and cortical neurons (52). Hypoxia dramatically upregulates Hif-1α expression in all brain cells including neurons, astrocytes and endothelial cells (53, 158, 54). Hif-1α accumulation occurs rapidly in rats exposed to hypoxia (10%O_2) and remains upregulated for 14 days. Notably, protein levels begin to decrease by 21 days (51, 53,157).

A recent study by Curristin et al., (55) using a rodent model of postnatal hypoxia shed light on the processes that are vulnerable in the hypoxic developing brain. A diagram of the most important changes reported in this study is illustrated in Fig. 4. In summary, the most dramatic changes involved genes required in the formation and stabilisation of mature and functional synapses. Interestingly, cytoskeletal proteins required for structuring neuritic processes were also largely reduced (for a more detailed description of the role of the cytoskeleton in neuronal responses see Section 4.4). Another set of genes that were greatly upregulated, were Hif target genes, and in particular VEGF with a known role in neuronal differentiation (52, 56).

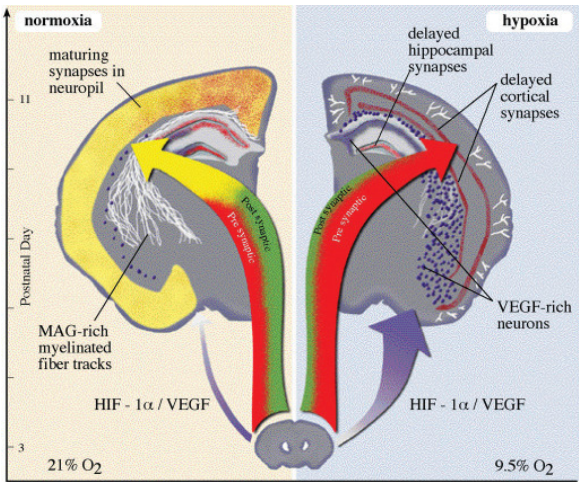


Fig.4. Hypoxia in the postnatal rodent brain leads to alterations in genes involved in maturation of synaptic transmission. Interestingly expression of genes involved in presynaptic function was exaggerated by hypoxia (red), while genes regulating postsynaptic function were largely decreased. A marked downregulation of cytoskeletal proteins was also observed. At the same time Hif-1α and Hif target genes were upregulated. (Figure taken from (55).

4.3.2 Age-dependent susceptibility to hypoxic/ischemic injury.

A great deal of experimental work shows that the immature brain is more resistant to hypoxia than the mature brain. This is reflected by evidence that acute hypoxia will very rarely damage the fetal or neonatal brain unless it is superimposed with ischemia (57, 58). The influence of age on hypoxic/ischemic brain injury is further supported by a number of studies which showed enhanced vulnerability of adult rats to hypoxia/anoxia in comparison to their younger counterparts (59, 60, 141, 61, 62). Although it is believed that all individuals have an inherent capacity for functional recovery, it is certain that the young brain is able to recover better than the adult mature one, a characteristic which is attributed to enhanced plasticity of the young brain (63, 64). A recent study by Yager et al., (63) based on behavioural tests, reported a greater degree and more rapid recovery of immature animals (10-day-old) than their mature counterparts following ischemic brain injury. The molecular mechanisms responsible for such differences are far from understood however it is

suggested that enhanced neurogenesis, synapse proliferation and activity-induced increase in synaptic connections are all mechanisms associated with increased survival and repair after injury (65, 66).

Elucidating the molecular pathways linked to increased resistance of the developing brain is crucial to our understanding of brain injury. However, care should be taken since several studies have reported that the immature brain is differentially regulated by various insults compared with the adult, due to the involvement of age-dependent molecular pathways. For example the differential roles of NMDA receptors in the immature brain as opposed to the adult one is worth mentioning since it underlines the importance of careful validation of therapeutical strategies for patients of different ages. In the developing brain, NMDA receptors have unique characteristics that allow them to open more easily and block less frequently than the mature ones, hence contributing to the increased brain plasticity observed during development. At the same time however, following a severe hypoxic/ischemic insult the neurons of the developing brain might be at a higher risk (67, 68). It was thus initially thought that drugs against the NMDA receptors would improve outcome following ischemic/hypoxic injury in the developing brain. However it is now known that drugs which block NMDA receptors following injury trigger widespread apoptosis in the developing brain, while at the same time are therapeutical in the adult one (69). These data indicate that developmentally regulated factors can indeed underlie age-specific differences in response to injury.

4.3.3 Stroke

As already mentioned hypoxia is an important pathological component of many disorders including stroke. It is additionally known that hypoxia differentially affects the various brain cells.

There are two main causes of stroke: i) a sudden decrease in oxygen levels, for instance as a consequence of respiratory arrest or ii) disruption of blood flow to the brain, which is caused either due to an occlusion or rupture of a blood vessel (ischemic or hemorrhagic stroke respectively). Disruption of blood flow deprives the cells from oxygen and nutrients, thus eventually leading to cell death. A schematic representation of stroke is shown in Fig.5. It is now established that hypoxic/ischemic injury involves multiple events, including oxidative stress, mitochondrial dysfunction, extracellular glutamate accumulation and inflammatory processes (70). The induction and coordination of such events is very complex, and the impact of stroke on the brain not only depends on the cell type, but also the age of the patient (as already described in the previous section), and the affected area.

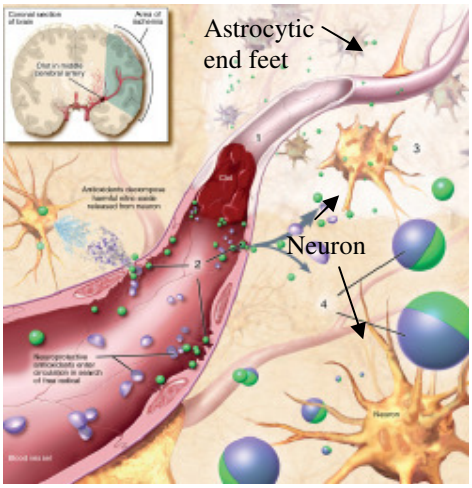


Fig.5. Schematic representation of stroke. Disruption of blood flow deprives cells from oxygen and nutrients. At the same time, free radicals are released that cause damage to endothelial cells and neurons and eventually lead to cell death. (Figure taken from: www.sfn.org BrainFacts).

Following an ischemic insult, neurons are the first brain cells to be affected. The neuronal response to hypoxic/ischemic injury results in overstimulation of the glutamate receptors namely NMDA and AMPA. More specifically, energy deprivation leads to an excessive release of glutamate, leading to hyper-activation of its receptors, accumulation of intracellular Ca^{2+} and ultimately neuronal death (71, 72).

The differential vulnerability of neurons as a result of age has been established and a number of hypotheses have been proposed. Kass et al., (73) suggested that immature neurons have greater intracellular ATP stores, which allow them to withstand longer periods of energy deprivation. Other reports indicated that the increased metabolic demands of mature neurons are partially responsible and contribute to the increased vulnerability of these neurons (74). More recently it was suggested that the reduced ability of mature neurons to reduce energy consumption accounts for the increased death (75). Indeed it is likely that a combination of these processes account for the age-dependent neuronal vulnerability to ischemic insults.

Neurons in different brain regions have characteristics that render them differentially vulnerable to an ischemic insult. In the preterm neonatal brain, the periventricular white region is the most vulnerable region (76) as opposed to the term brain where basal ganglia are known to be the most susceptible (77). Additionally, hippocampal neurons start dying already after 5 min of global brain ischemia (78) while cortical neurons are more resistant, death being observed after 15-20 min . The mechanisms of selective vulnerability in the face of a global insult are not understood, but several *in vitro* studies have provided important information. A study by Jiang et al., (79) suggested that insufficient antioxidant defences in combination to excessive pro-oxidant production might account for the higher vulnerability of immature

hippocampal neurons to oxygen-glucose deprivation *in vitro.* Several other studies have indicated that regional differences in repair capacity (80) and regulation of genes controlling apoptosis (81) might also account for differential vulnerability.

On the other hand, astrocytes are far more resistant to hypoxic/ischemic injury, a phenomenon that has been linked to their greater anti-oxidant capacity. *In vitro* studies have revealed that glutathione, a major intracellular anti-oxidant, is present in higher amounts in astrocytes compared to neurons (72). It should however be noted that vulnerability of astrocytes also depends on the brain region. For example rat hippocampal astrocytes are more vulnerable to oxygen-glucose deprivation than cortical ones, similar to data from neurons (81).

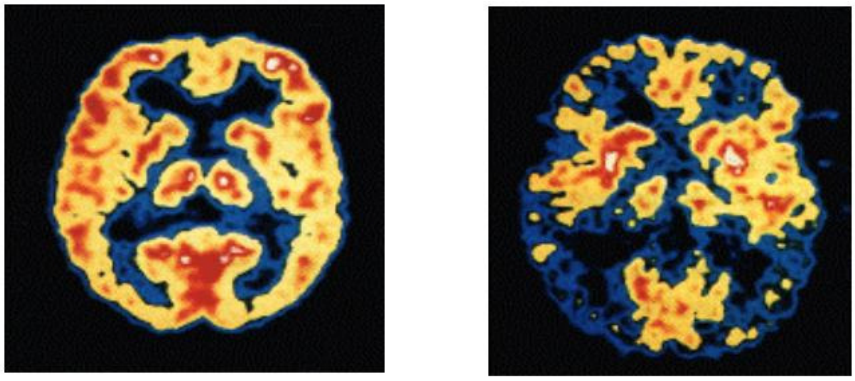
Furthermore, astrocytes support neuronal function and survival through their ability to take up glutamate and release trophic factors (82-84). For instance, it has been shown that Epo and VEGF protein secretion in the media of hypoxic astrocytes is far more robust compared with neurons, a response which is thought to serve as a trophic support for the latter (85). However, at the same time astrocytes contribute to brain damage, by retracting their end feet from vessels thus increasing permeability of the blood brain barrier, as well as by proliferation giving rise to a glial scar (86, 87).

The fact that astrocytes are extremely resistant to hypoxia, together with their important role in supporting neuronal survival renders them particularly interesting in the filed of stroke. Understanding the molecular mechanisms that are employed by these cells and render them more resistant to other brain cells can provide important clues about hypoxic tolerance and protection against injury.

4.3.4 Alzheimer's disease and Hypoxia

Hypoxia is implicated in the pathogenesis of a number of neurodegenerative disorders such as Alzheimer's and amyotrophic lateral sclerosis (AMS) (4, 88). These neurodegenerative disorders are characterised by accumulation of potentially toxic proteins. It is thought that proteins which are present in the organism throughout life-time become neurotoxic as a result of environmental stimuli. Amongst others hypoxia is suggested as a causative agent contributing to neurodegeneration (89, 90).

The prevalence of Alzheimer's disease is directly related to age and represents a huge healthcare issue for the society. Approximately 90,000 people in Switzerland suffer from Alzheimer's, and the number is expected to rise further in the next 20 years. Currently the cost of care for patients with Alzheimer's around the world is approximately $150 billion annually. Alzheimer's patients show severe memory and cognitive function impairment, which is believed to be the outcome of disrupted neuronal circuits and severe neuronal loss. Fig.6. indicates the great loss in neuronal activity in the brain of an Alzheimer patient.



Normal Alzheimer

Fig.6. Positron emission tomography scans show differences in brain activity between a brain of a healthy individual and a brain of an Alzheimer patient. Blue and black zones indicate inactive areas. (Figure taken from www.ipa-online-org)

The neuronal cytoskeleton has a key role in the pathogenesis of Alzheimer's. The pathological hallmarks of Alzheimer's disease are large extracellular plaques composed of β−amyloid (Aβ) and intraneuronal neurofibrillary tangles (NFTs) composed of abnormally hyperphosphorylated forms of the microtubule-associated protein tau. The accumulation of plaques and tangles as the disease progresses leads to extensive neuronal and synaptic loss. According to the amyloid hypothesis, progressive accumulation of Aβ in the brain initiates a cascade of events that includes oxidative injury, metabolic alterations and initiation of signaling pathways that will eventually lead to the breakdown of the cytoskeleton and cell death. Injection of Aβ into the brain of mice expressing a mutant form of human tau, leads to accelerated NFT formation (91). Additionally, in cultured primary neurons, application of Aβ peptides leads to increased tau phosphorylation and cell death (92). A number of proteins have been shown to be involved in tau hyperphosphorylation. Amongst them the protein kinases cyclin-dependent kinase 5 (cdk5) and glycogen synthase kinase 3 (GSK3) are key regulators of these processes (93, 94). Cdk5 is of particular interest in this study, since its activity is downregulated with neuronal age, but at the same time it increases in certain disease situations, such as ischemia and Alzheimer's (see section 4.5). Deregulation of cdk5 by Aβ *in vitro* induces an increase in tau phosphorylation (95, 96). In addition, increased cdk5 expression is present in neurons exhibiting NFT and it has been shown to physically interact and phosphorylate tau *in vivo* therefore strongly suggesting induction of cdk5 by Aβ may lead to tau phosphorylation (97, 98).

Interestingly, the presence of Aβ in the cerebrospinal fluid (CSF) of healthy individuals and also in the media from neuronal cultures suggests a physiological role for Aβ (99). It is therefore argued that Aβ becomes toxic only when the balance

between its production and degradation is disturbed. Although Aβ accumulation is most probably caused by more than one mechanism, it is thought that environments characterized by a decrease in energy metabolism, such as a hypoxic/ischemic environment, probably contribute to Alzheimer's pathogenesis. In fact, individuals who have suffered severe hypoxia or ischemia are more likely to develop Alzheimer's (100). Further support for this hypothesis was provided by experimental evidence showing that hypoxia enhances accumulation of the Aβ peptide (101). In addition an interesting study by De la Monte et al., (102) revealed that hypoxia leads to neuronal loss with neuritic sprouting, impaired mitochondrial function and reduction in expression of proteins that are required to maintain synaptic connections. The authors concluded that since similar abnormalities characterize Alzheimer's neurons, hypoxic injury is likely to contribute to Alzheimer's neurodegeneration.

Thus, although the molecular mechanisms are far from being understood, it appears that exposure to hypoxia is indeed involved in and accelerates neurodegeneration.

4.3.5 Hypoxic preconditioning in the brain.

The protective effects of adaptation to hypoxia have been demonstrated with respect to diseases of both the cardiovascular system and the CNS. *In vivo* studies have revealed that subjecting animals to a moderate hypoxic/ischemic insult activates a "defence" mechanism which protects the animal against a subsequent severe insult, a situation known as pre-conditioning (49, 103, 104). In concomitance, exposure of neurons to hypoxia can be protective against a more severe insult *in vitro* (105-107) Arthur et al., 2003). It is believed that Hif pathways and in particular the Hif target gene EPO, which is commonly expressed in neurons and astrocytes are potential mediators of hypoxic preconditioning (108-110). Treatment of neurons with Epo

immediately prior to hypoxia protects neurons from subsequent death (111, 112). Similarly, Epo treatment was neuroprotective in an *in vitro* model of ischemia, i.e. oxygen/glucose deprivation (113). Interestingly, Epo treatment did not protect astrocytes in this model, indicating a cell specific effect.

Notably, pre-conditioning has been proposed as an alternative therapy not only for stroke but also for some neurodegenerative disorders, such as Alzheimer's disease. According to some scientists Alzheimer's disease is the outcome of exhausted adaptive reserves in the brain and thus therapeutic strategies should aim to enhance those reserves and prevent deterioration of brain cells. Several lines of evidence suggest that adaptation to hypoxia may indeed serve this role (114). Adaptation to hypoxia prior to Aβ injection in rats prevented memory impairments (114). Additional evidence for the potential protective effects of hypoxic adaptation on Alzheimer's disease come from recent experimental work showing that induction of Hif-1α pathways in neurons *in vitro* protects neurons from Aβ neurotoxicity. Last but not least, Chong et al.,(115) provided evidence for a neuroprotective role of Epo against Aβ toxicity in hippocampal neurons *in vitro*. More specifically Epo treatment was sufficient to prevent Aβ induced apoptotic death both in early but also in later stages of neurodegeneration.

Therefore understanding the molecular mechanisms in the brain's response to hypoxia is not only important for understanding disease pathogenesis but also for the development of new therapeutical strategies against numerous diseases.

4.4 THE CYTOSKELETON AS A MODULATOR OF THE HYPOXIC RESPONSE

The cytoskeleton is a structure composed of three classes of proteins: microtubules (tubulin), microfilaments (actin) and intermediate filaments. The three classes of cytoskeletal proteins have unique properties, which are attributed to their distribution. In neurons (the main focus of this study), microtubules are present throughout the cell and are mainly involved in intracellular transport but also in neuritic elongation. Actin microfilaments are most abundant in presynaptic terminals, dendritic spines and growth cones and play a more important role in sprouting, and axon pathfinding (Fundamental Neuroscience, 1999). It is worth mentioning that actin is also involved in proper localisation and function of vital proteins such as Na^+/K^+-ATPase but also cell adhesion proteins such as the cadherins (116, 117). Intermediate filaments, known as neurofilaments, are present along the axons and their function is generally been considered to be structural (Fundamental Neuroscience, 1999). Naturally cytoskeletal proteins also work synergistically and together contribute to maintain cell shape, produce locomotion, provide mechanical strength and facilitate the intracellular transport of organelles. Such processes are critical to the proper development and function of an organism but also in disease state, for instance in the ability of a tumour cell to become metastatic and also in CNS diseases characterised by cytoskeletal disruption.

As already mentioned in previous sections, communication between neurons, and therefore information processing, is largely accomplished between axons of presynaptic neurons and dendrites of postsynaptic neurons. Maintenance of axonal and dendritic morphology, which is largely dependent on cytoskeletal proteins, is thus crucial for normal neuronal function. Understanding how the cytoskeleton is

regulated in response to environmental cues is a major challenge. To date, a large number of proteins are identified that have a regulatory role in cytoskeletal dynamics. Of those the Rho GTPase family, which also participates in cell adhesion, migration and gene transcription, has received a lot of attention (118). In neurons the cdk5/p35 complex plays a major role in coordinating cytoskeletal dynamics, most probably also through its interaction with members of the Rho GTPase family (119-121).

It is now widely accepted that cytoskeletal alterations, as a result of external stimuli, lead to changes in protein interactions and signalling. To date we know that a number of disease states are characterised by cytoskeletal re-arrangements that might culminate in cell death. Alterations in the actin cytoskeleton have been linked with neurodegeneration in diseases such as Alzheimer's (122) (and section 4.3). As mentioned above actin microfilaments are mainly present in neuritic dendrites and dynamic regulation of actin polymerisation is implicated in maintaining dendrite structure. It was subsequently shown that the number of dendritic spines is significantly decreased in Alzheimer's neurons, indicating an active role for actin in disease progression (123). Additionally, actin-rich inclusions known as Hirano bodies, have been described in Alzheimer's disease but also in other neurodegenerative disorders such as Niemann Pick's disease (124, 125).

The involvement of the cytoskeleton in the cellular hypoxic response has been reported in several studies. Hypoxic-induced disorganisation and abnormal distribution of F-actin has been shown in endothelial cells, vascular smooth muscle cells and also in neurons (126-128). Additionally, in neuronal cells, hypoxia induces the dissociation of actin from membrane associated proteins such as spectrin and ankyrin (117). Other studies have reported that anoxia leads to the breakdown of neurofilaments, and dephosphorylation of the microtubule-associated protein tau.

(129, 130). Conversely, ischemia/reperfusion in rats leads to tau hyperphosphorylation, as a result of aberrant cdk5 activation (131). The above evidence indicate an important role for the cytoskeleton in the neuronal hypoxic response. Recently, the role of the cytoskeleton and/or cytoskeleton regulatory proteins in hypoxic signalling pathways and more specifically in Hif regulation was documented (132, 133). Turcotte et al., (132) reported that F-actin disruption by cytochalasin upregulates pVHL expression, a key protein in Hif-1 regulation in kidney cells (see section 4.1.1). Additional studies by the same authors showed regulation of Hif-1 by RhoA in renal carcinoma cells exposed to hypoxia. In Hep3B cells Hif-1 hypoxic regulation involves Rac1, another member of the Rho GTPase family (134). A more recent study by Shen et al.,(133) reported that the neurotrophin receptor binding protein, MAGE-D1 (melanoma-associated antigen family protein-D1), interferes with actin cytoskeleton organisation and subsequently regulates Hif-1 expression in HeLa cells. A major focus of this study was to understand whether similar mechanisms are employed by neurons following a hypoxic insult.

4.5 CDK5/p35, A MODULATOR OF THE NEURONAL CYTOSKELETON.

Cdk5 is a 33kDa protein serine/threonine kinase that belongs to the family of cyclin dependent kinases due to its close sequence homology to cdk2 (135, 136) (Fig. 7). Interestingly, cdk5 does not seem to be involved in cell cycle processes and its activity is not regulated by cyclins (1, 137). Instead, cdk5 activity is regulated upon direct binding to its activators p35 and p39 (Fig.8), and their proteolytic fragments p25 and p29 respectively (138-142). Neither p35 nor p39 share any detectable sequence similarity with the cyclins. As opposed to other cyclins, cdk5 does not require phosphorylation of the catalytic subunit on the activation loop for activity

(Morgan et al., 1997). It is shown that binding of cdk5 to its activators is sufficient to fully induce kinase activity (143, 144). However, phosphorylation of the residues Ser^{159} and Tyr^{5} can also increase cdk5 activity (119, 145).

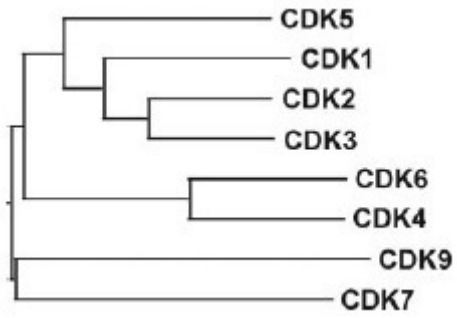


Fig.7 A dendrogram of human cyclin-dependent kinases showing the close sequence homology between cdk5 and other cdks (Figure taken from(1)).

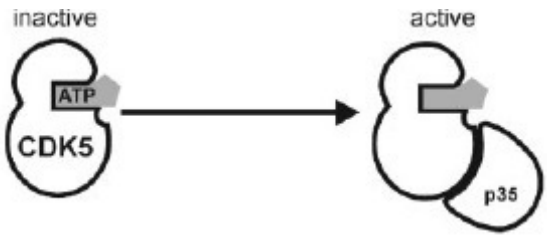


Fig. 8. Association with either p35 or p39 is sufficient to fully induce cdk5 activity (Figure taken from (1). Binding of either p35 or p39 induces conformational changes to cdk5, which allows the kinase to bind to its substrates.

Although cdk5 is widely distributed in mammalian cells, its activity is restricted to the brain due to the localisation of its activators (146-148). In the developing brain, cdk5 expression increases gradually to reach maximal levels in post-mitotic neurons. More specifically, in rodents cdk5 initially appears in young neurons at embryonic day 12 (E12) and reaches a peak at postnatal day P7 (137). Instead, cdk5 activity reaches a peak at P14 after which it begins to decline (149). A recent study by Lu et al., (150) reported a decrease in p35 mRNA expression in aged individuals, further indicating downregulation of cdk5 activity with age.

The biological function of the cdk5/ p35 complex has been studied extensively (151-154). *In vitro*, both cdk5 and p35 are detected throughout the neurites and the neuronal soma. However a fraction of p35 and cdk5 is also shown to localise in the nucleus (155-157). Nowadays, evidence has accumulated for the contribution of cdk5 in a number of neuronal functions, including cytoskeleton regulation, axonal guidance, cell signalling and synaptic plasticity (121, 158-160). The substrate specificity of the cdk5/p35 complex requires the K(S/T)PX(K/R) motif where S or T (serine or threonine respectively) are the candidate residues for phosphorylation (161-163). Initially, cdk5 was described as a neuronal cdc2-like kinase that phosphorylates the KSP motif of neurofilaments (NFs) (135, 164, 165). However, it is now accepted that the cdk5/p35 complex is fundamental in brain development through interaction with a variety of cytoskeletal proteins that include synapsin I and the actin filaments (166, 167). Interestingly there is increasing evidence for a role of the complex in the adult brain. For example, cdk5 activity is linked to dopamine signalling in the striatum (168), and also in NMDA receptor clustering in the adult mouse brain (169). Additionally deregulation of cdk5 activity contributes to a number of neurodegenerative disorders, by hyperphosphorylation of the microtubule-associated protein tau, and neurofilaments (170-172). Some selected examples of known cdk5 substrates during development and in the adult brain are summarised in Fig.9.

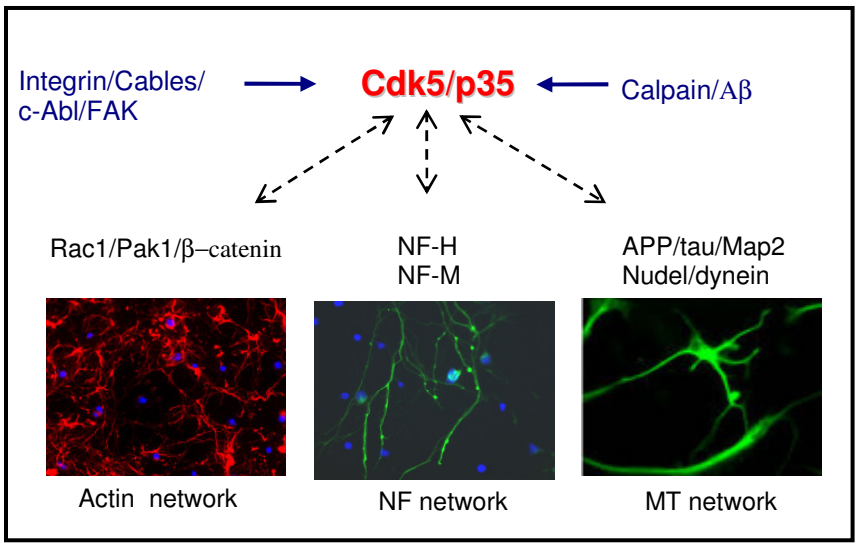


Fig.9. Examples of some activators (blue) and effectors (black) of the cdk5/p35 and their role in the regulation of the neuronal cytoskeletal networks: actin, neurofilaments and microtubules. Proteins are categorised according to which neuronal network they are most likely to be associated. Note that the complex can also directly regulate cytoskeletal proteins, such as NF-H and NF-M. Images were obtained in our lab.

4.5.1 Cdk5 in neuronal death and survival

It is believed that cdk5 can act as a pro-survival or pro-death protein depending on the type and duration of the stimulus, as well as on the age and type of the neuron (152, 173, 174). Cdk5-deficient mice die before or just after birth with severe defects in the cortex and other compartments of the central nervous system while p35-deficient mice, although viable and fertile, display seizures and sporadic lethality. Immunohistochemical analysis of these mice brains reveals that the lamination pattern of the cerebral cortex is disrupted, underlying the role of cdk5 in neuronal migration (175, 176). The role of cdk5 in regulating developmental neuronal pathways is further highlighted by the fact that neuronal apoptotic death induced by UV radiation is enhanced in the absence of cdk5. More specifically, Li et al (177) showed that cdk5 attenuates activation of c-Jun N terminal kinase 3 (JNK3) following UV radiation by directly phosphorylating JNK3 and thereby increasing survival. Generally it is

believed that under neurotoxic conditions, proteolytic cleavage of p35 by calpain results in the generation of a more stable fragment, p25, which leads to cdk5 mislocalisation, and deregulation of its activity (178-180). In neurodegenerative disorders, deregulation of cdk5 activity is linked to disruption of the cytoskeleton and eventually neuronal death (181, 182). *In vitro* studies reveal that introduction of p25 in primary neurons leads to neurite retraction, microtubule collapse and apoptosis (181). It is thus thought that cdk5 translocation as a result of p25 production, allows the kinase to interact with alternative substrates and contribute to apoptosis. More specifically the cdk5/p25 complex is shown to directly phosphorylate tau *in vitro* and contribute to neurodegeneration (183), while p35/cdk5 does not (181).

It was initially believed that cdk5 requires p35 to exert its pro-survival functions, while binding to p25 renders it pro-apoptotic. Recent evidence however seems to contradict this hypothesis, and suggest that p25 production is not detrimental per se, but can contribute to neuronal death only when chronically present. For instance, Fischer et al., (160) reported that transient p25 expression facilitated hippocampus-dependent memory in mice, while prolonged p25 expression on the other hand was tightly associated with cognitive deficits and neuronal death.

It appears that cdk5 regulation is complex with distinct functions depending on the stimulus and also on the age of the cell. The elucidation of additional signalling pathways and thus of novel molecular players that interact with cdk5 is crucial in order to fully understand the different functions of this kinase and was addressed in this study.

4.5.2 Cdk5 in hypoxic/ischemic injury

The first evidence for a role of cdk5 in ischemic injury came by Green et al.,(184). Using a rat model of complete ischemia the authors reported a transient increase in cdk5 activity in the brain. Additionally, increased p35 levels and cdk5 activity have been reported in neurons after middle cerebral artery occlusion (185) but also in human stroke tissue (186) implicating the cdk5/p35 complex in the neuronal response to ischemic injury. Notably, inhibition of cdk5 activity attenuated infarct size in models of brain ischemia (187), (188), and dominant negative cdk5 expression protects against damage in a model of focal stroke *in* vivo (189). Although the exact mechanisms by which cdk5 facilitates neuronal death are not fully understood there are data suggesting that cdk5 can phosphorylate and thus directly modulate transcription factors involved in neuronal survival/death pathways. For instance, deregulated cdk5 can phosphorylate and inhibit the protective effects of myocyte-enhancing factor 2 (MEF2) that is induced by excitotoxicity (190) Cdk5 also phosphorylates and thus increases the activity of p53, a pro-apoptotic transcription factor (191). Interestingly, in neurons oxygen deprivation and more specifically Hif-1 is an inducer of p53 (37), however a link between cdk5 and these molecules as a result of hypoxic injury is not yet identified.

Conversely, cdk5 might be vital in recovery or protection from neuronal injury. For instance, cdk5 activity is vital for the neuroprotective effects of aspirin against rat spinal cord hypoxia/reoxygenation injury *in vitro* (192). Also prenatal hypoxia in rats results in delayed neuronal migration and downregulation of cdk5 (193). Although the data are not conclusive, the authors suggested that cdk5, being an important player in neuronal migration, might be responsible for the observed hypoxic-induced changes. Interestingly O'Hare et al., (194) reported that cytoplasmic cdk5 is neuroprotective in

models of excitotoxicity and DNA damage *in vitro*, whereas nuclear cdk5 is harmful and attributes to neuronal excitotoxic death.

The above evidence indicate a role for the cdk/p35 complex in ischemic injury, however little is known about the role of the complex in oxygen deprivation alone, a question that was directly addressed in this study.

5 CHARACTERISATION OF CELLULAR AND MOLECULAR MECHANISMS IMPLICATED IN NEURONAL RESPONSE TO HYPOXIA

5.1 **Objectives**

Structural and functional integrity of the brain requires proper neuronal function and viability, which in turn rely on a constant oxygen and glucose supply. Disruption or alterations in oxygen availability can therefore be life threatening and may lead to irreversible brain damage. Hypoxia characterises a number of physiological events such as exercise and high altitude exposure as well as contributing to disorders of the CNS such as stroke, head trauma and neurodegenerative disorders. Therefore adaptation to hypoxia is essential for life and involves complex mechanisms of sensing and responding at the systemic, local, cellular and molecular level.

At the cellular level, disruption of the actin cytoskeleton is a hallmark of the hypoxic response (195). Nowadays the importance of the cytoskeleton has been reviewed (196) and it is believed to play an active role in signal transduction, upon a stressful stimulus. Cytoskeletal alterations as a result of hypoxia not only affect the cellular morphology but also lead to changes in protein interactions ultimately affecting survival. At the same time, at the molecular level, sensing and responding to hypoxia involves stabilisation of Hif-1 and consequently transcription of a number of gene products that are involved in cellular metabolism (5, 11, 197). Although the molecular mechanisms involved in Hif-1 regulation have been extensively studied, recent reports point to the involvement of the cytoskeleton and/or cytoskeleton regulatory proteins in Hif-1 regulation. A large number of proteins with a role in regulating the neuronal cytoskeleton have been identified (198), amongst them the cdk5/p35 complex (119). The important role of this complex in neuronal survival in several

excitotoxic models renders it a possible candidate as a regulator of the neuronal hypoxic response.

The aim of this work was to expand our basic understanding of neuronal responses to hypoxia by elucidating novel molecular mediators. In particular we were interested in understanding the involvement of the F-actin cytoskeleton and cytoskeletal-regulating proteins in neuronal hypoxic responses. We hypothesised that the cdk5/p35 complex, a key regulator of the neuronal cytoskeleton (see section 4.5), is modulated by alterations in oxygen level, and in its turn is involved in hypoxic regulation of the F-actin cytoskeleton. Additionally, we sought to understand how hypoxic-induced cytoskeletal alterations might affect signal transduction and eventually neuronal survival. Based on literature evidence for the role of the actin cytoskeleton in Hif-1 regulation (see section 4.4), this study focused on the possible involvement of the p35/cdk5 complex as part of a molecular mechanism contributing to the regulation of Hif-1 signalling pathways in hypoxia.

5.2 **Own Research**

5.2.1 Hypoxic modulation of the cdk5/p35 complex

A number of reasons led us to hypothesise that the cdk5/p35 complex is modulated by hypoxia. In *Drosophila*, anoxia up regulates the expression of p35 (199). Additionally, a study by Green et al., (184) reported a transient increase in cdk5 activity in a model of irreversible brain ischemia in the rat. Since then a number of studies have shown modulation of the cdk5/p35 complex by excitotoxicity (181, 194, 200, 201).

In order to understand the role of the cdk5/p35 complex in the neuronal hypoxic response, we used an *in vitro* model of primary neurons isolated from mouse

embryonic brain (embryonic day E14). Neurons were cultured for 6 days (6DIV) and were exposed to 1%O_2 for different periods of time. We showed that following hypoxia both cdk5 and p35 mRNA levels are transiently increased. In concomitance, cdk5 activity was transiently increased and paralleled the increase in p35 protein levels. Notably, both p35 protein levels and cdk5 activity fell to below normoxic levels by 24h, coinciding with a dramatic decrease in ATP levels and a significant increase in neuronal death. Additionally, immunocytochemical analysis revealed that cdk5 localisation is also affected by hypoxia, with a clear translocation of the protein to the neuronal soma. Our results, together with some recent scientific evidence showing interaction of cdk5 with transcription factors (190) suggest that under certain conditions the kinase is translocated and can interact with alternative targets. Although hypoxia did not affect p35 localisation, we observed a change in the electrophoretic mobility of the protein upon hypoxic exposure.

Altogether, our results clearly and for the first time show that oxygen deprivation alone regulates the cdk5/p35 complex *in vitro* and indicated a role for the complex in the neuronal hypoxic response.

5.2.2 Cdk5 modulates the F-actin cytoskeleton during hypoxia.

Disruption of the F-actin cytoskeleton as a result of cellular responses to hypoxia has been documented in a number of studies (see section 4.4). It is believed that reduced oxygen levels lead to changes in the neuronal shape, which are partially attributable to depolymerisation of actin filaments. In neurons, in addition to the Rho GTPase family, the cdk5/p35 complex has an established role in the modulation of the cytoskeleton. Thus we hypothesized that hypoxia can cause F-actin re-arrangements that are regulated by the cdk5/p35 complex. To test our hypothesis, primary neurons

were pretreated with the ckd5 inhibitor roscovitine and were exposed to hypoxia for 2h, 4h and 6h. Consequently, changes in the F-actin cytoskeleton were observed by immunocytochemistry and compared to normoxic and hypoxic controls.

Results show that hypoxia effected F-actin organization, reflected by retraction of the F-actin filaments, in a time dependent manner. Thus, in neurons, similar to other cells, disruption of the F-actin cytoskeleton is an early event in response to hypoxic injury. Cdk5 inhibition by its pharmacological inhibitor roscovitine also resulted in an earlier disruption of the F-actin cytoskeleton upon hypoxia but not in normoxic. Thus cdk5 participates in modulation of the neuronal hypoxic response by maintaining the F-actin cytoskeleton.

5.2.3 Cdk5 modulates Hif-1α signalling pathways and neuronal survival in chronic hypoxia.

The neuronal hypoxic response is regulated to a large extent by the transcription factor Hif-1 (202, 203), which in its turn is regulated by variety of proteins contributing to its stabilization and activation (see section 4.1.1, Table 1). A number of recent studies have reported the involvement of proteins that regulate the cytoskeleton in Hif-1 regulation (132, 133). We have shown that cdk5 is involved in modulating Hif-1α hypoxic induction. Our hypothesis and observations are supported by previous reports where it has been shown that serine/threonine inhibitors can significantly inhibit Hif-1 hypoxic induction (204).

Indeed hypoxia induces Hif-1 expression, which in turn correlated with elevated cdk5 activity. Notably, cdk5 inhibition attenuated accumulation of Hif-1 during hypoxia, with a dramatic down regulation observed after 24h. As expected, hypoxia also led to a significant up regulation of neuronal levels of the Hif target genes, Epo and VEGF.

Interestingly, roscovitine application did not significantly affect VEGF mRNA levels but led to a dramatic down regulation of Epo mRNA levels, indicating differential modulation of Hif-1α signalling pathways by cdk5. Taken together our results show for the first time, that cdk5 participates in the neuronal hypoxic response via modulation of Hif-1 induced neuroprotective pathways. The involvement of cdk5 in regulation of Hif inducible pathways indicates an important role for the kinase in neuronal survival following hypoxia. To test this hypothesis, roscovitine was applied to neuronal cultures before hypoxic exposure, and neuronal death was assessed by measuring LDH release in the medium. Interestingly, significant cell death was observed after prolonged but not acute periods of hypoxia and this response was exacerbated in the presence of the cdk5 pharmacological inhibitor, roscovitine, thus providing evidence that cdk5 activity is required for neuronal survival following a prolonged hypoxic insult. Moreover our results indirectly point to the importance of Epo signalling in neuroprotection, since its inhibition by roscovitine coincides with the increased cell death observed in neurons exposed to hypoxia and roscovitine treatment.

In summary, cdk5 is an important player of the neuronal hypoxic response. Inhibition of the kinase attenuates Hif-1 signalling pathways and consequently enhances the neuronal vulnerability to hypoxia.

5.3 VARIATIONS IN THE HYPOXIC RESPONSES OF MATURE NEURONS

Dissociated primary cultures are a simplified yet valuable model allowing the easy evaluation of molecular and cellular responses to a specific stimulus. Primary neuronal cultures exposed to hypoxia can thus be considered simplified models of cerebral hypoxia that enable detailed investigation of hypoxic regulated pathways and provide insights into disease pathogenesis. Additionally, it is now widely accepted that neurons developing *in vitro* and *in vivo* share important characteristics such as developmental specific susceptibility to neurotoxicity and maturation-dependent regulation of death/survival pathways (205). In fact, several *in vitro* studies have shown increased vulnerability to hypoxia with neuronal age, supporting the general believe that the developing brain is more resistant to hypoxic injury than the adult one.

In this study we aimed to pinpoint differences in the early events that characterize the neuronal hypoxic response as a result of maturational stage.

The cdk5/p35 complex is developmentally regulated and seems to have different roles depending on the age of the neuron. In development, the complex is involved in neuronal migration and differentiation, while in the mature nervous system it is involved in maintaining neuronal plasticity (206-208). We have already shown that the cdk5/p35 complex is regulated by hypoxia in immature neurons, and is involved in modulation of the F-actin cytoskeleton and Hif-regulating pathways, ultimately affecting neuronal survival. We next hypothesized that the cdk5/p35 is differentially regulated by hypoxia in mature neurons. Thus the role of the complex in modulation of the F-actin cytoskeleton and Hif signaling pathways was further investigated.

For the purpose of this study and in order to identify maturational-dependent hypoxic responses, primary neurons were cultured for 17 days (17DIV, mature) before hypoxic exposure.

5.3.1 Hypoxia dramatically reduces ATP levels and disrupts the F-actin cytoskeleton.

Mature neurons responded to hypoxia with a dramatic drop in ATP levels (Fig. 1). Hypoxia resulted in a 90% decrease in ATP levels already after 2h ($P<0.001$) however, increased exposure times had no additional effect on ATP levels suggesting that in mature neurons energy supplies fall to critical levels very early in response to hypoxia. Previous reports have documented that the ATP content of a cell in part defines the state of actin filaments. It is further reported that approximately half of neuronal ATP is consumed by cycles of actin polymerisation-depolymerisation (195). Since mature neurons respond to hypoxia with a dramatic drop in ATP levels, we assessed the impact of hypoxia on F-actin organisation. Immunocytochemistry analysis with TRITC-phalloidin, which stains the F-actin cytoskeleton, revealed a well-defined and intense phalloidin staining in neuronal dendrites of normoxic cultures (Fig. 2a). In contrast, exposure of neurons to 1% O_2 resulted in a marked disruption of the F-actin cytoskeleton, as revealed by retraction of the actin filaments (Fig. 2b-c). These results indicate that alterations of the F-actin cytoskeleton together with the dramatic drop in ATP levels are early events characterising the response of mature neurons to hypoxia.

5.3.2 Hypoxia induces both necrotic and apoptotic death.

Reduced ATP levels are closely linked to cell death. To determine vulnerability of mature neurons to hypoxia, cell death by necrosis was assessed using the LDH assay. Fig.3A shows that hypoxia induced a time-dependent increase in cytotoxicity of 23.7%±4.2 ($P < 0.01$) and 36.07%±6.4 ($P < 0.01$) compared to normoxic values after 6h and 24h respectively, suggesting that hypoxia rigorously induces death of mature neurons. To investigate whether apoptotic processes were initiated, we assessed caspase-3 activation, a key marker of apoptosis. A 1.56±0.06 ($P < 0.01$) fold increase in caspase-3 activity was observed after 6h (Fig. 3B) providing evidence for induced apoptotic neuronal death in this model. Time course analysis suggested that caspase-3 activity was sustained, although not further induced after 72h of hypoxia.

Thus we can conclude that hypoxia leads to increased neuronal death which involves both necrotic and apoptotic processes in mature neurons, as opposed to immature neurons that do not show any signs of apoptotic death even after 24h of hypoxia.

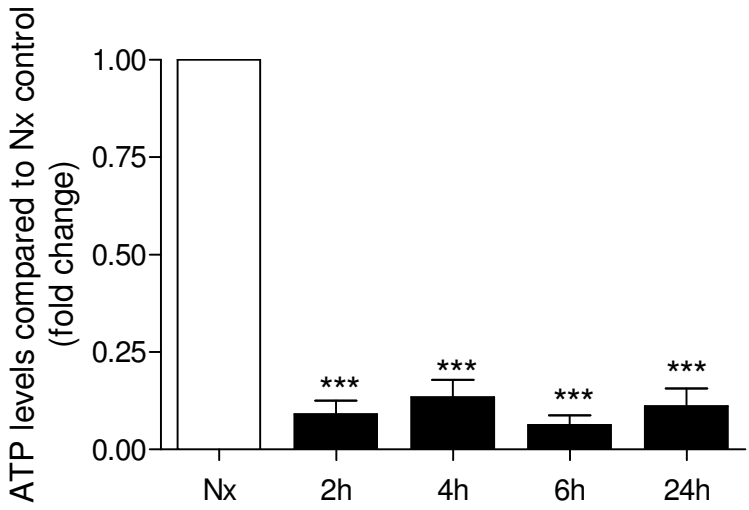


Fig. 1. ATP levels are severely depleted after 2h of hypoxia ($P<0.001$) 2h of hypoxia lead to a dramatic 90% decrease in ATP levels compared to normoxia. Increased exposure times have no additional affect.

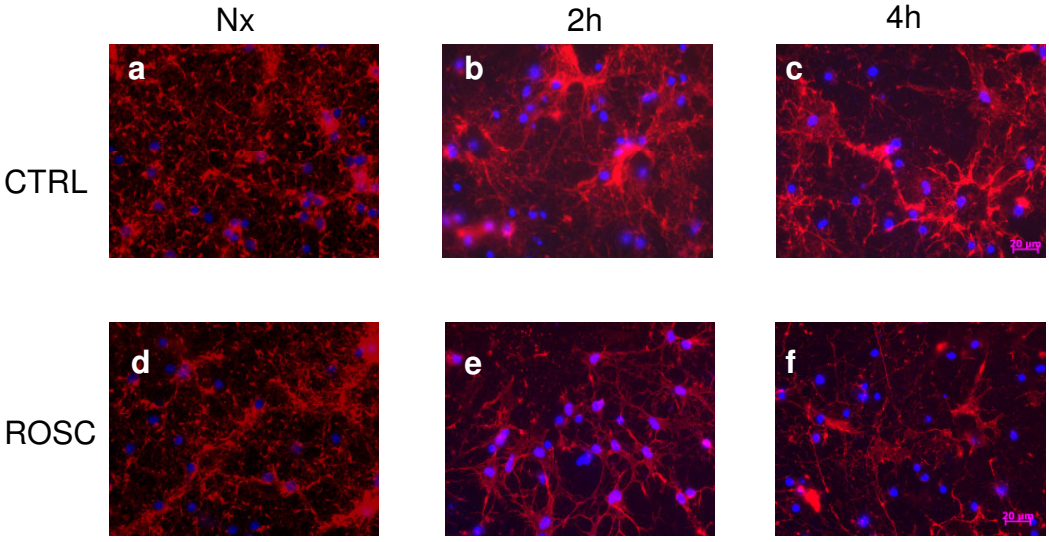


Fig. 2. Cdk5 inhibition exacerbates F-actin retraction induced by hypoxia. Cortical neurons were treated with 20µM roscovitine and were then exposed to 1%O_2 for 2-4h and double stained for nuclei (DAPI, blue) and F-actin (phalloidin-TRITC, red). a) Representative figure demonstrating intense F-actin dendritic staining in normoxic neurons. b-c) Hypoxia leads to retraction of F-actin fibers. Cdk5 inhibition with roscovitine (ROSC) did not affect F-actin organisation in normoxia (d), but exacerbated F-actin retraction in the hypoxic cultures (e-f).

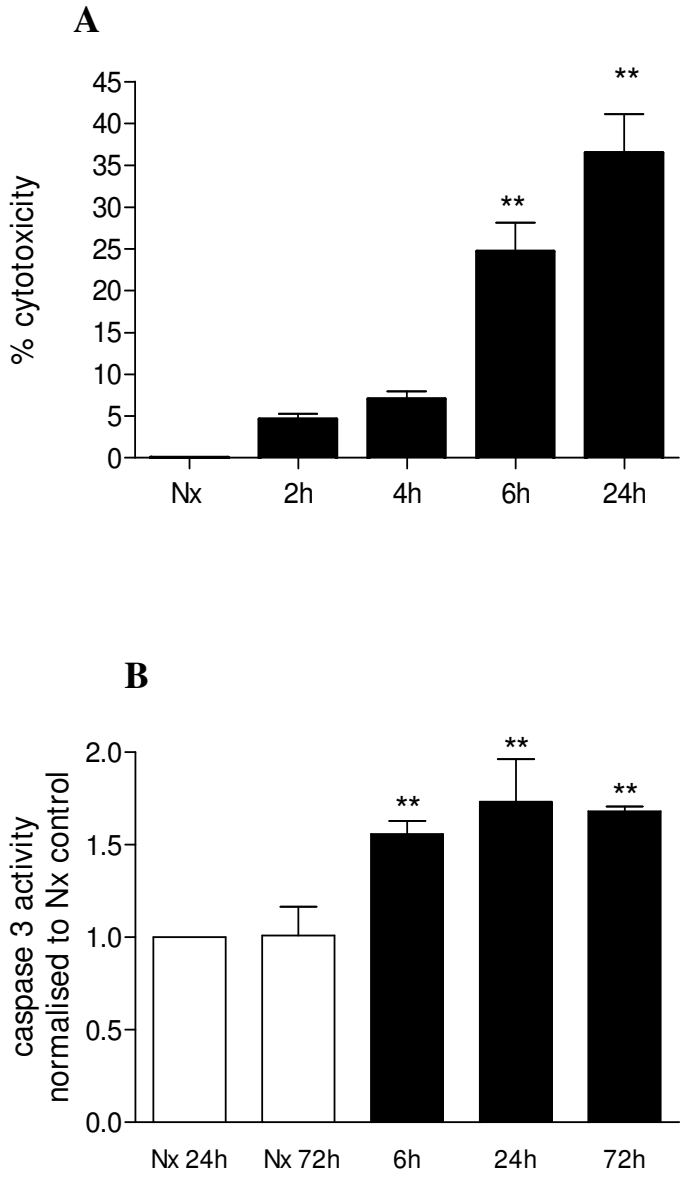


Fig. 3. Hypoxia leads to an increase in cell death. A) An increase in neuronal death was demonstrated by the increased release of LDH in the medium. B) Increased caspase-3 activity is observed after 6h and indicates the initiation of apoptotic processes.

5.3.3 Hypoxia induces p35 cleavage.

In the adult brain deregulation of the cdk5/p35 complex is tightly interconnected with the progression of neurodegenerative disorders such as Alzheimer's disease. Under neurotoxic conditions, proteolytic cleavage of the cdk5 activators, p35 and p39, by calpain results in the generation of p25 and p29 respectively, leading to mislocalisation of cdk5, deregulation of its activity and cytoskeletal disruption.

In light of the pivotal role played by cdk5/p35 complex in adult-brain neurodegenerative diseases and the similar events characterising Alzheimer's disease and hypoxia (see section 4.3.4), we hypothesised that cdk5/p35 complex participates in differential hypoxic responses of mature neurons. Modulation of the complex was assessed at both the transcriptional and translational level. As shown in Fig. 4A hypoxia significantly up regulated p35 mRNA levels after 4h (34.43 ± 1.18, $P<0.001$). This increase was transient and mRNA levels returned to baseline after 6h. Western blot analysis showed downregulation of p35 expression with increased exposure times. (Fig.4B). Interestingly, p25 was formed by 2h although p35 was still present, unlike in young neurons where hypoxia led to p35 dephosphorylation but not to p35 cleavage. The presence of p25 in 17DIV neurons following hypoxic exposure is indicative of calpain activation, initiation of cell death pathways, and cdk5 deregulation. By 24h both p35 and p25 levels decreased below normoxic values suggesting cdk5 activity downregulation.

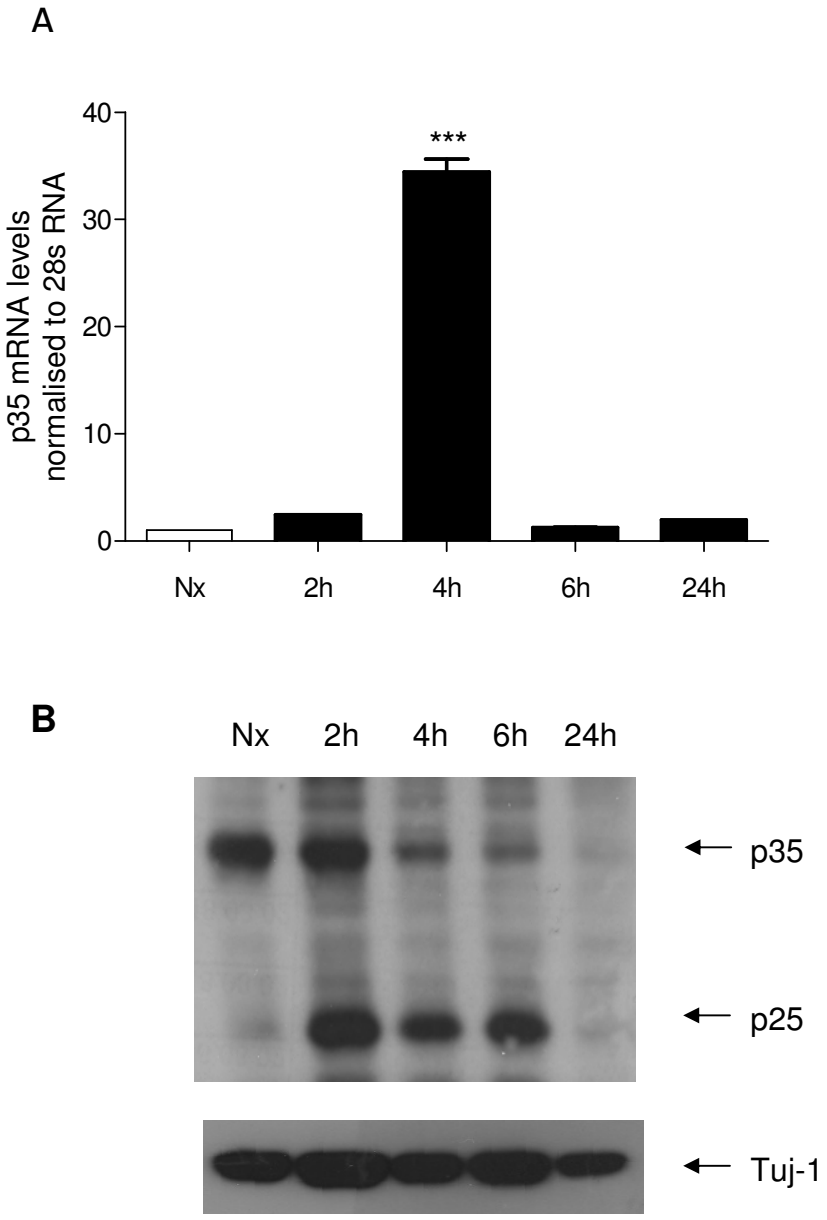


Fig. 4. p35 is modulated by hypoxia both at the mRNA and protein level. A) RT-PCR (Real-Time PCR) analysis shows that p35 mRNA is transiently induced by hypoxia. Values were normalized to 28s RNA and normoxic controls. B) Western blot analysis shows modulation of p35 and p25 levels following hypoxia. Note that p35 is cleaved to its proteolytic fragment p25 after 2h. Both p35 and p25 fall below normoxic levels after 24h.

5.3.4 Cdk5 activity is transiently increased by hypoxia.

To determine if cdk5 is a potential candidate for regulation of survival in response to hypoxia, we examined the effect of hypoxia on cdk5 mRNA and protein levels. As shown in Fig. 5A, mRNA levels were significantly increased following 4h (14.57±0.28, $P<0.001$) but returned to normoxic levels after 6h. Conversely, cdk5 protein levels were not affected by hypoxia (Fig. 5B).

The presence of p25 following exposure of neuronal cultures to 2h of hypoxia suggested deregulation of cdk5 activity. Thus, the effect of hypoxia on cdk5 activity was determined by *in vitro* kinase assays using Histone H1 as a substrate, where phosphorylation of Histone H1 was a direct measurement of cdk5 activation. Cdk5 activity increased at 2h and remained high up to at least 6h, coinciding with the increase in p25 levels (Fig. 5C). Interestingly, cdk5 activity was reduced below normoxic levels after 24h. Additionally, phosphorylation of cdk5 on Serine 159, as assessed by western blot analysis, was also increased after 4h, further underlying modulation of cdk5 by a hypoxic stimulus (Fig. 5D). Notably, the decrease in cdk5 phosphorylation and activity at 24h coincided with a 47.17% neuronal death suggesting a possible role for the kinase in cell survival.

Altogether our results show that hypoxia regulates cdk5 activity in both immature and mature neurons. However the presence of p25 only in mature neurons would suggest that the mechanisms which contribute to cdk5 activity regulation are different in different maturational stages.

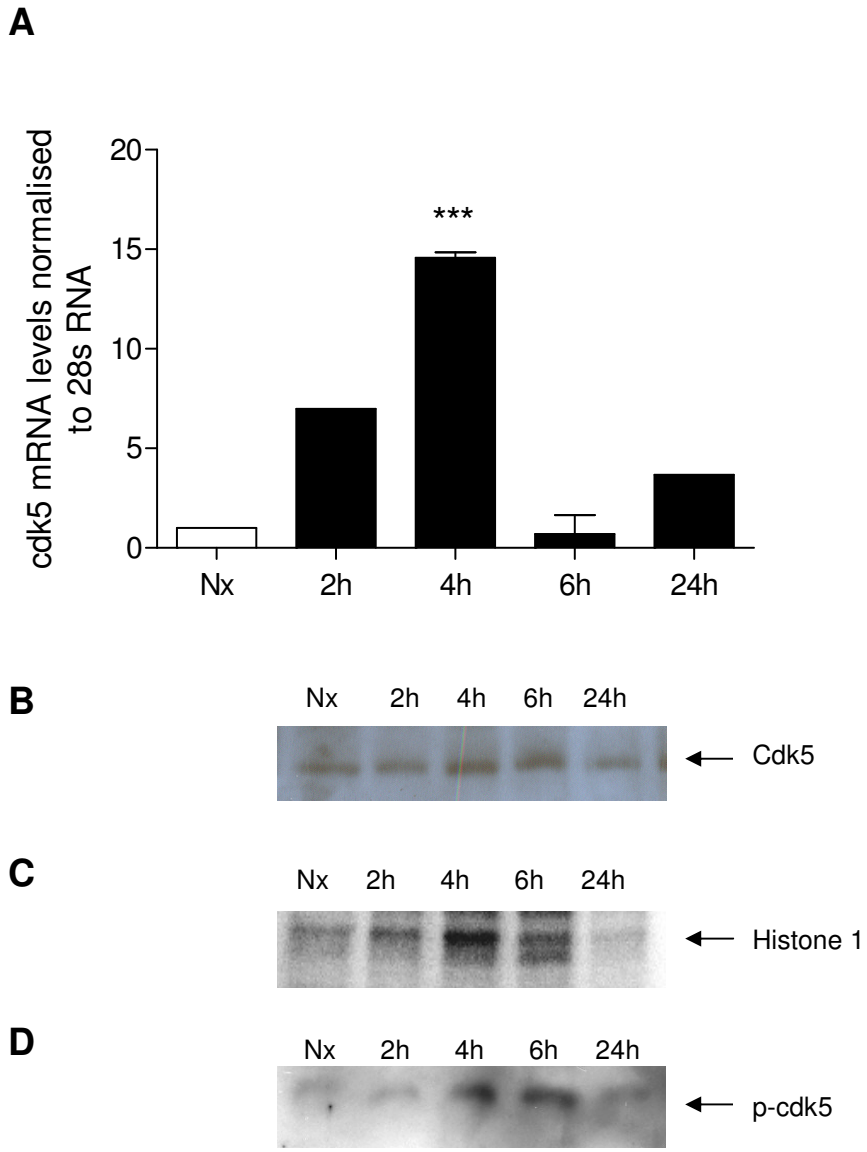


Fig.5. Hypoxia transiently induces cdk5 mRNA levels and activity. A). RT-PCR demonstrates increased cdk5 mRNA expression after 4h hypoxia. B) Cdk5 protein levels are not affected by hypoxia. C) *In vitro* kinase assay of immunoprecipitated cdk5 using Histone 1 as a substrate shows that cdk5 activity is greatly induced by hypoxia between 2-6h and returns to normoxic levels by 24h. D) Western blot analysis shows a gradual increase in cdk5 phosphorylation of the Ser^{159} residue.

5.3.5 Cdk5 inhibition exacerbates F-actin disruption following hypoxia.

Roscovitine is a purine analogue that has been described for its function as a potent inhibitor of cdk5 (Vita et al., 2003). In our study we used 20μM of roscovitine.

To determine whether cdk5 is involved in cytoskeletal re-arrangements that accompany hypoxia, neuronal cultures were pretreated with roscovitine prior to hypoxic exposure. As shown in Fig. 2d, roscovitine pre-treatment did not affect the F-actin network in normoxic cultures. However, addition of the drug to hypoxic cultures led to a further retraction of the F-actin fibers when compared to hypoxic controls (Fig. 2 e-f). This retraction was time dependent, suggesting that cdk5 has a pertinent role in maintaining the F-actin cytoskeleton during hypoxic injury. Since a similar effect was observed in immature hypoxic neurons, we conclude that this is a universal mechanism employed by neurons in response to hypoxia.

5.3.6 Cdk5 inhibition attenuates Hif-1 hypoxic induction and binding activity.

A number of recent reports indicated the involvement of proteins, such as the Rho-GTPases, that interfere with F-actin re-arrangements in the regulation of Hif-1 induction (132, 134). Since cdk5 regulated the F-actin cytoskeleton following hypoxia, and actin-regulating proteins are involved in hypoxic Hif-1 regulation, we hypothesized a possible role for cdk5 in Hif-1 regulation at the protein level. In order to test our hypothesis induction of Hif-1α levels were determined in hypoxic neurons after roscovitine treatment. Fig. 6A shows attenuation of Hif-1 expression after 6h of hypoxia and roscovitine application.

The effect of cdk5 inhibition on Hif-1 activity was further assessed by EMSA analysis (Fig. 6B). As expected no Hif-DNA binding complex was detected under normoxic conditions however hypoxia-inducible binding activity was observed after 4h and

further increased after 6h (Fig. 6B, lanes 3, 5). When roscovitine was applied to the neuronal cultures, Hif-binding activity was markedly inhibited (Fig. 6B, compare lanes 3 and 5 to lanes 4 and 6 respectively), suggesting that cdk5 can modulate Hif-1 binding activity during a hypoxic stimulus.

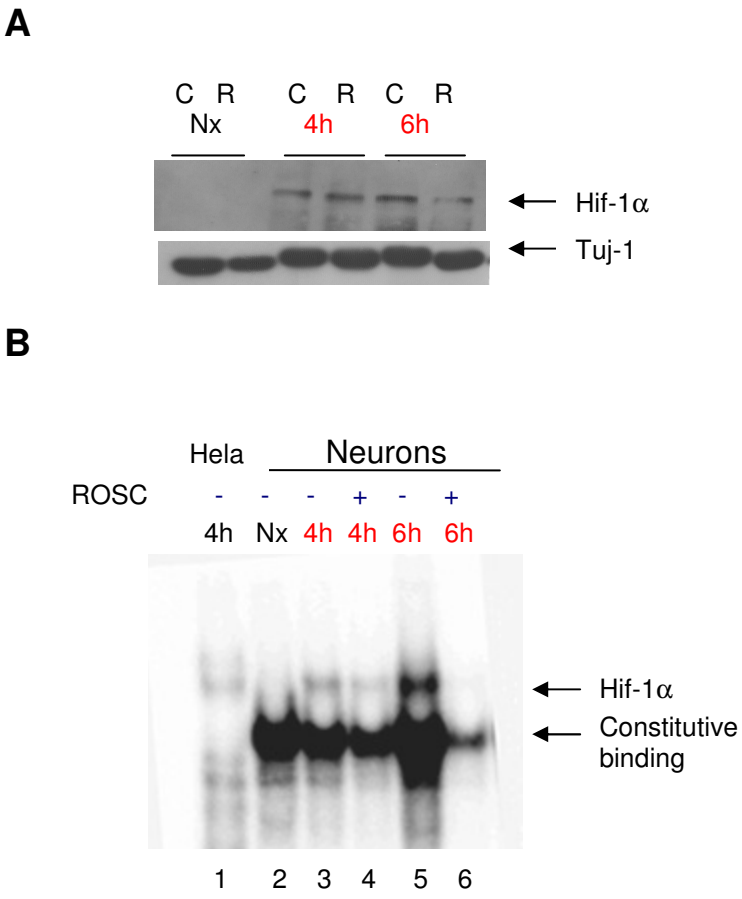


Fig. 6. Cdk5 inhibition attenuates Hif-1α hypoxic induction and binding activity. A) Western Blot analysis shows that roscovitine inhibits Hif-1α hypoxic induction after 6h. C= control, R= 20μM Roscovitine B) Representative EMSA showing Hif-1α hypoxia inducible binding activity in mature neurons after 4h and 6h (Lane 3 and 5 respectively). Roscovitine inhibits Hif-1α binding activity already by 4h. Nuclear extracts from Hela cells exposed to hypoxia were used as a control.

5.3.7 Cdk5 inhibition differentially regulates Hif-1 signalling pathways.

Since cdk5 inhibition attenuates Hif-1α accumulation and binding activity, the effect of cdk5 inhibition on the regulation of Hif-1 inducible neuroprotective pathways was investigated. For this purpose Epo, VEGF and GLUT1 mRNA expression levels were assessed by RT-PCR. As expected, hypoxia induced mRNA expression of all three genes in a time-dependent manner (Fig. 7A-C). Surprisingly, roscovitine application did not significantly affect VEGF mRNA levels at any time point (Fig. 7A). Similarly, roscovitine did not affect GLUT1 levels although after 24h of hypoxia a tendency to reduced GLUT1 mRNA levels was observed (Fig. 7B). On the other hand and most intriguingly, application of the cdk5 inhibitor led to complete abolishment of Epo mRNA induction (Fig. 7C). The effect of roscovitine in abolishing Epo induction was already observed after 4h and was present for the rest of the hypoxic experiment. Altogether these results show that cdk5 differentially regulates Hif-1 neuroprotective pathways and additionally indicate a role for the kinase in determining neuronal survival following a hypoxic insult.

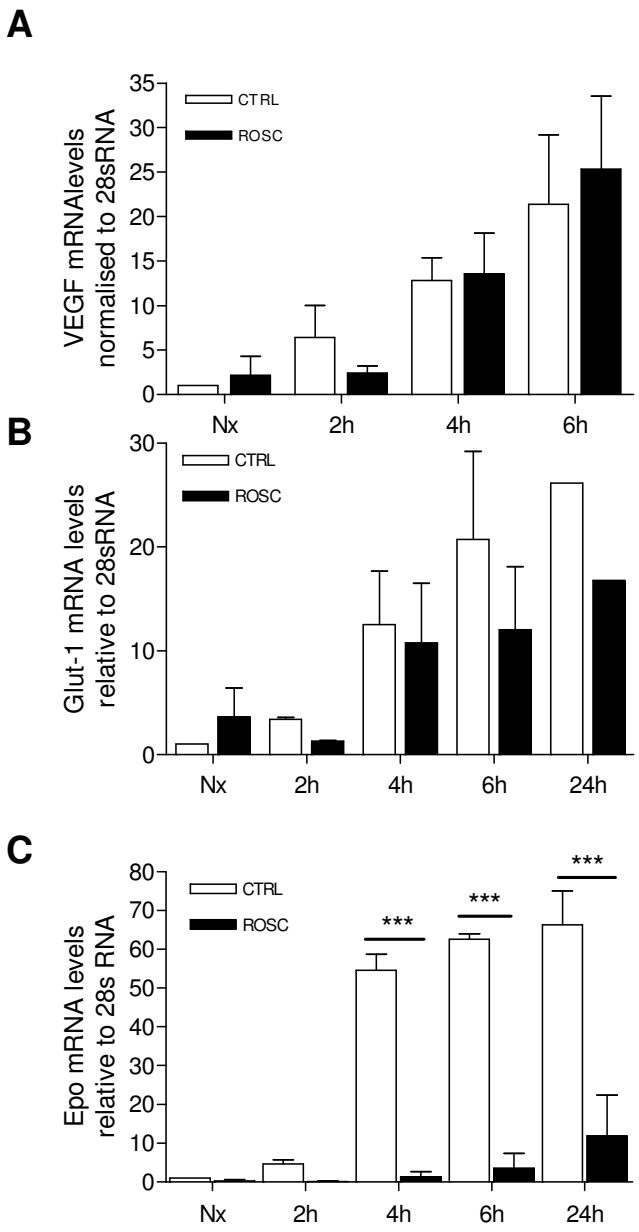


Fig. 7. Cdk5 inhibition differentially regulates Hif target genes during hypoxia. The effect of roscovitine application on Epo, GLUT1 and VEGF mRNA levels were assessed by RT-PCR. A) Roscovitine treatment has no effect on VEGF mRNA levels. B) A tendency to reduced GLUT1 mRNA levels is detected following roscovitine application and 24h of hypoxia. C) Roscovitine rapidly abolishes hypoxic-induced Epo mRNA expression after 4h. ($P < 0.001$). All values are representative of 3 independent experiments and are normalised to 28sRNA and normoxic controls.

5.3.8 A role for cdk5 in prolonged hypoxic injury?

The involvement of cdk5 in regulation of Hif-1 inducible pathways that have been shown to be neuroprotective indicated a role for the kinase in neuronal survival. In mature neurons 24h of hypoxia led to 44.31% increase in cell death compared to normoxic neurons, as assessed by LDH assay. Surprisingly roscovitine application for 24h did not significantly alter hypoxic induced neuronal death, suggesting that cdk5 inhibition does not have a role in acute hypoxia (Fig. 8). Preliminary results suggest that inhibition of cdk5 might exacerbate cell death in later time points, indicating a role for the kinase in chronic hypoxia. Interestingly in immature neurons, roscovitine inhibition also enhanced cell death only in chronic hypoxia, thus further underlying a role of the cdk5 kinase in chronic injury.

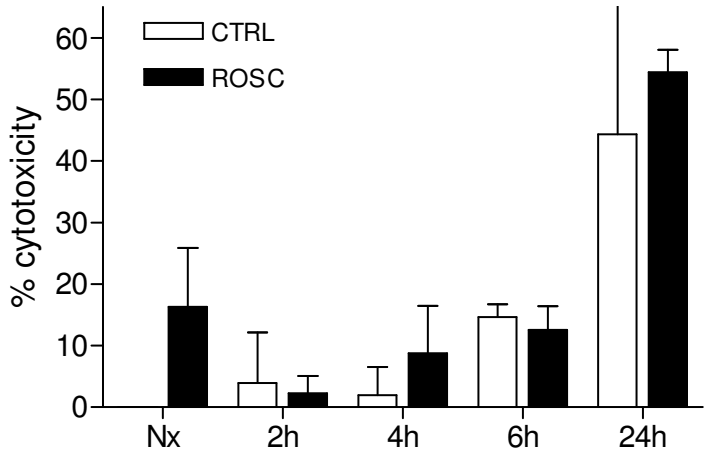


Fig. 8. Roscovitine application does not significantly affect neuronal survival following 24h of hypoxia, as assessed by LDH assay.

5.4 **Discussion I:** Cdk5 and the neuronal hypoxic response

The mechanisms employed by the organism and determine the outcome of hypoxic/ischemic brain injury as a result of age remain largely unknown. Nevertheless, laboratory work suggests that in fetuses and neonates hypoxia is unlikely to cause brain injury unless superimposed with ischemia (Reviewed by (57). Further support is provided by a number of *in vivo* studies which have shown that younger animals survive hypoxia/anoxia better than adults (59, 60), reported that vulnerability of the rat brain to hypoxic/ischemic brain injury increases during development, especially between postnatal days P7 and P15. Additionally *in* vitro studies have underlined the increased vulnerability of neurons to excitotoxic insult as they mature over days *in vitro* (209), (210, 211). Our results are in agreement with others, showing that hypoxia alone has a more severe effect in mature neurons and support the belief that immature neurons are resistant to hypoxic injury. Since our *in vitro* data parallel results obtained in *in vivo* studies, such an *in vitro* model system is valuable for elucidating the molecular and cellular mechanisms of the neuronal response to hypoxia and can be employed for studying development-regulated pathways.

Generally, hypoxia/ischemia can lead to cell death that is either necrotic or apoptotic or both. Initiation of both necrotic and apoptotic processes have been described in models of ischemic injury both during development and in adult models (211, 212). However in our *in vitro* model, apoptosis was not initiated in young neurons, suggesting that the hypoxic stimulus could be tolerated. Interestingly, necrotic cell death was observed in both immature neurons and mature neurons by 6h, however the latter were more severely affected. In most cells, initiation of necrotic death coincides with a decrease in cellular ATP contents to greater than 80-85%, although the

threshold can vary in different cell types (213). In fact increased cytotoxicity in mature neurons correlated with the more dramatic drop of ATP levels. Conversely, immature neurons were able to maintain reduced but constantly higher ATP levels for a longer period of time (6h) than mature ones. Our studies agree with other reports showing that the better maintenance of ATP levels in immature neurons contributes and provides longer protection against anoxia (75). Kass et al., (73), had previously suggested that immature neurons have greater intracellular ATP stores, which allow them to withstand longer periods of energy deprivation. However in our studies intracellular normoxic ATP stores were similar in both maturational stages (data not shown), suggesting that rather the increased metabolic demands of mature neurons, or their inability to reduce energy consumption most probably accounted for their increased vulnerability. Whether these neurons have reached "a point of no return", where mitochondrial impairment is irreversible irrespective of any intervention (i.e. reoxygenation) is an interesting question that arises from these observations and will be addressed in the future. However, we speculate that mature neurons with lower hypoxic resistance and the earlier exhaustion of energy stores, reach this point earlier.

Several reports in the field have attempted to identify the cellular changes that accompany cellular ATP depletion and culminate in cell death. Two hypotheses have been proposed: i) ATP concentrations below a certain level hinder phosphorylation of proteins required for cell survival, ii) ATPase-dependent cellular functions are impaired as a result of low ATP levels and cytoskeletal disruption. To date, several studies support the impact of decreased ATP levels on the F-actin cytoskeleton in the hypoxic response. We, in agreement with others (193), show that hypoxia leads to disruption of the F-actin cytoskeleton and a decrease in total levels of F-actin. This disruption has severe implications in neuronal function. Direct comparison of the

hypoxic-induced F-actin disruption between the two maturational stages could not be assessed with the experiments performed in this study, but we remain convinced that the faster exhaustion of ATP levels in mature neurons together with the observed F-actin disruption partially contributes to increased vulnerability.

The cdk5/p35 complex controls many different pathways via phosphorylation of a wide array of proteins (Section 4.5). and it has been shown to influence a variety of neuronal function including: i) neuronal migration in development (159, 214, 215) ii) synaptic plasticity in mature neurons (174, 206, 216) and iii) neuronal death in neurodegenerative disorders (217-219). Our results for the first time show an age-dependent regulation of the cdk5/p35 complex by acute hypoxia. In immature neurons hypoxia led to a change in the electrophoretic mobility of p35 indicating dephosphorylation. At present the reasons why the phosphorylation state of p35 change are unknown, however previous reports have linked p35 dephosphorylation to decreased ATP levels (220), thus agreeing with our data showing that dephosphorylation of p35 coincides with a drop in ATP levels following hypoxia. Conversely, in mature neurons hypoxia led to the proteolytic cleavage of p35 and formation of the shorter fragment, p25. Our data agree with those of others showing that in the mature neuron dephosphorylated p35 is susceptible to calpain cleavage and leads to p25 production (182, 220). Numerous studies have shown that under neurotoxic conditions calpain-induced p35 cleavage results in the generation of p25 leading to mislocalisation of cdk5 and deregulation of its activity (182, 221). Our results indicate that only in mature neurons hypoxia leads to calpain activation, further underlying the increased susceptibility to hypoxia with age. The absence of p35 cleavage in immature neurons suggests that at least for the duration of the

hypoxic stimulus studied calpain pathways are not initiated. Interestingly, hypoxia led to a transient increase in cdk5 activity in both maturational stages. Thus our results support the idea that both p35 and p25 can upregulate cdk5 activity following a stress stimulus. Notably it has also been shown by others that oxidative stress and serum deprivation *in vitro* are stimuli which lead to deregulation of cdk5 activity in the absence of p25 (200, 222). Moreover maintenance of cdk5 in these experimental models had a protective role against neuronal toxicity.

During this study, a number of hypotheses were proposed about the differential roles of the cdk5 activators in determining cdk5 function. It was initially suggested that p35 is required for the pro-survival roles of cdk5 and on the other hand p25 is the factor determining the cdk5 pro-death functions. A number of studies provided evidence for the above hypothesis (Section 4.5). Now, scientists realise that the picture is more complicated. Fischer et al., (160) showed that in the adult mouse transient upregulation of p25, not only is not detrimental for the cell, but is actually beneficial in memory-related processes. In contrast, prolonged p25 expression caused severe cognitive deficits and neuronal loss allowing the authors to conclude that in aged individuals, p25 production only contributes to neurodegeneration if chronically present. In a more recent study, Anne et al., (223) reported that cdk5 activity is transiently activated following DNA damage and protects neurons from death, but prolonged DNA damage leads to loss of cdk5 activity and enhanced cell death. In a similar manner we observed a biphasic regulation of cdk5 activity following hypoxia. Thus we also propose that cdk5 activity increases as an early response to hypoxic injury, however at later time points cdk5 contributes to cell death. Indeed prolonged hypoxia led to a decrease in cdk5 activity in both maturational stages which coincided with increased cell death. Additionally and particularly in immature neurons, chronic

hypoxia (36h-48h) together with application of the cdk5 inhibitor roscovitine significantly induced death suggesting a specific role for cdk5 in modulation of neuronal survival pathways during long-term hypoxic injury/insult. In mature neurons, hypoxia alone is sufficient to lead to a dramatic increase in cell death. Interestingly, a recent study by Lu et al., (150) showed that in the human adult brain (40 years) expression of the cdk5 gene is reduced. It is possible that downregulation of the cdk5/p35 complex in aged neurons contributes itself to the observed increased vulnerability in adult neurons. Thus, it is tempting to suggest that shared mechanisms may exist between aging and hypoxic injury. Further *in vivo* studies are required to support this hypothesis.

Although we have not identified the exact mechanisms that lead to the transient increase in cdk5 activity, it is possible that it was induced as a result of the disruption of the F-actin framework observed following hypoxia. In fact it has been shown that cytochalasin D, leads to a drastic re-organisation of the F-actin cytoskeleton but also causes an increase in cdk5 activity in COS7 cells transfected with p39, another cdk5 activator (167). The authors further reported cytochalasin D-induced redistribution of p39 protein to F-actin clusters and suggested that availability of the cdk5 activator is the limiting factor determining cdk5 activity. A supportive study by Lee et al., (224) showed that the neuronal cdk5/p35 complex associates with a kinase inhibitor protein in the form of a macromolecular complex and that certain stimuli can destabilise the complex and lead to an increase in the availability of the cdk5 activators, p35 and p39 ultimately leading to an increased kinase activity. Indeed we believe that hypoxia might be such a stimulus, but further studies are required to examine this possibility.

Interestingly, inhibition of cdk5 led to an earlier disruption of the F-actin cytoskeleton upon hypoxia, indicating an active role for the kinase in hypoxic-induced F-actin re-

arrangements in both ages. We therefore propose a feedback loop, by which hypoxic-induced alterations of the F-actin cytoskeleton induce cdk5 activity, which in turn regulates the dynamics of the F-actin filaments. Disruption of the actin architecture may involve the Rho GTPase family members RhoA and Rac1, which are also involved in the regulation of the actin cytoskeleton and their activity is tightly regulated by hypoxia in several cell types (225-228). Interestingly, the intracellular distribution of these proteins is different in neurons of different maturational stages. In immature neurons, during the period of axon and dendrite sprouting, both Rho members are distributed evenly, while in mature neurons RhoA is predominantly found in dendrites and Rac1 in axons (229). Although we do not have direct evidence, the fact that in immature neurons Rac1 interacts with p35 in a GTP-dependent manner, together with our preliminary data showing that Rac1 inhibition exacerbates neuronal death only in hypoxic immature neurons, would suggest that Rho GTPases might indeed participate with the cdk5/p35 complex in the neuronal hypoxic response but that these interactions might differ depending on the age of the neuron. Further studies in this field are required to elucidate whether and how the Rho-GTPases interact with the cdk5/p35 complex and together contribute to the modulation of the F-actin cytoskeleton as part of the neuronal response to hypoxia.

At the molecular level, the neuronal hypoxic response is regulated to a large extent by the transcription factor Hif-1 (202, 203). In all neurons, increased cdk5 activity correlated with elevated Hif-1 expression and binding activity. The involvement of the cytoskeleton and/or cytoskeleton-regulating proteins in Hif-1 modulation has been a recent matter of discussion (see section 4.5). We show for the first time that cdk5, a neuronal specific cytoskeletal regulator, is involved in the mediation of hypoxia-

dependent, Hif-1α-induced pathways in both mature and immature neurons. The next question to be addressed concerns the actual mechanisms by which cdk5 controls Hif-1. Does cdk5 directly interact and modulates Hif-1 by phosphorylation? Previous studies have shown that treatment of cells with a non-specific serine/threonine kinase inhibitor also blocks the hypoxia-induced expression of Hif-1α (204) suggesting that cdk5-mediated phosphorylations may be important in Hif-1α regulation. Notably, following database analysis, we have identified the presence of a putative phosphorylation site for cdk5 in the Hif-1α coding sequence. It is however also possible that cdk5 regulation of Hif is the outcome of cytoskeletal re-arrangements induced by cdk5 following a hypoxic stimulus. Several studies showing that cytoskeletal-regulatory proteins are involved in Hif-1α regulation (132-134), support this hypothesis. Further research is required to answer these questions.

Another interesting question that rises from these studies is whether and how Hif-1α availability in neurons changes with aging, and how such changes might affect neuronal survival upon hypoxia. To date reduced Hif-1α protein expression with increasing age has been reported in smooth muscle cells, rat cerebral cortex, mouse heart and carotid body (230-232) Data in our lab agree with those of others (232, 233) and show that Hif-1α levels are reduced in mature neurons compared to immature ones. Lower Hif-1 production could partially explain why cdk5 inhibition only had a minor contribution to hypoxic- induced death of mature neurons. The mechanisms involved in age-dependent Hif-1α reduction are unknown, however a recent report (Ndubuizu et al., Sfn meeting 2007) indicated a significant increase in the expression of the Hif-regulating proteins PHDs in old rats, suggesting that they probably contribute to the age-dependent modulation of Hif-1α expression.

Hypoxic-induction of Hif-1 enhances the transcription of a number of genes including VEGF, a known neuroprotective factor (26, 56). Surprisingly, cdk5 inhibition did not affect hypoxic-induction of VEGF mRNA levels suggesting that Hif-1 independent mechanisms might be responsible for hypoxic VEGF induction. Indeed our study on the astrocytic hypoxic response (see section 6.2.2) indicates that other mechanisms yet to be identified might be involved in VEGF regulation.

GLUT1 is another well described Hif target gene (13). Upregulation of GLUT1 by hypoxia has been reported in several studies and it is involved in induction of anaerobic ATP synthesis (30, 234). Our results show that GLUT1 mRNA levels were only significantly upregulated in mature neurons, suggesting an increased energy demand. In line with the above, we also detected decreased ATP levels in mature neurons which would consequently elicit upregulation of glycolysis that would in turn upregulate GLUT transporters. This is an interesting observation since it suggests not only differential regulation of Hif-pathways in different maturational stages but also underlines the increased susceptibility of mature neurons to hypoxia. Surprisingly, cdk5 inhibition did not significantly affect GLUT1 mRNA levels at least for the time points examined, indicating that cdk5 does not exert its functions through regulation of this Hif pathway.

One of the best characterised Hif-1α target genes is Epo, a key mediator of neuroprotection (11, 235, 236). Several *in vitro* models have shown that neurons exposed to hypoxia significantly upregulate Epo and mRNA and protein levels (237). In addition, pretreatment of neurons with Epo can prevent neuronal death in *in vitro* models of hypoxia and reduce ischemic damage in various animal models of stroke (72, 110, 238). As expected, Epo mRNA levels were upregulated by hypoxia in both maturational stages. In agreement with the observed inhibitory effect of roscovitine on

Hif-1, induction of Epo mRNA levels was significantly downregulated upon cdk5 inhibition. Altogether these results provide supplementary evidence that cdk5 is involved in the hypoxic neuronal response through differential modulation of Hif-1 signalling pathways and are in line with Chavez et al.,(85) where it was reported that in neurons Hif-1α binds preferentially to Epo as opposed to astrocytes where Hif-2α is preferentially binding to Epo. Moreover, in immature neurons our results are suggestive of the importance of Epo neuroprotection since its indirect inhibition by roscovitine coincides with increased cell death. On the other hand, in mature neurons cdk5 inhibition and subsequently abolishment of Epo does not seem to have an important contribution in hypoxic-induced neuronal death. We believe that in mature neurons hypoxia leads to maximal activation of cell death and thus inhibition of cdk5 has only a minor role in the destiny of these neurons.

5.5 Conclusions I and Outlook I

Hypoxia is implicated in the pathogenesis of a number of central nervous system disorders such as stroke and neurodegenerative diseases. At the same time, hypoxic preconditioning is protective against a more severe ischemic event. Other recent studies suggested adaptation to hypoxia as a potential defence mechanism for preventing CNS disorders (114). It is thus important to understand in which situations hypoxia is beneficial for the organism, and when this condition becomes detrimental.

The key factors defining the role of hypoxia seem to be duration, severity and age. Our data emphasise the impact of hypoxic injury in neurodegeneration of mature neurons and indicate that caution must be taken when considering adaptation to hypoxia as an alternative therapy for neurodegenerative disorders. The present work also provided a novel link between two crucial pathways, namely cytoskeletal rearrangements and hypoxic signalling, both of which have severe consequences on neuronal survival and disease progression. Indeed, a direct connection between cdk5, aberrant function of which leads to development of tauopathies, and Hif-1 the master regulator of adaptation to stress stimuli provides an important basis for future work. Our aim is to elucidate further not only the interaction of the cdk5/p35 complex with Hif-1, but also understand the contribution of the cdk5/Hif 1 pathway to the pathogenesis of neurodegenerative diseases.

A question that arises from our studies is how aging impacts on the brain vulnerability to injury, even though the molecular pathways that are initiated as a result of hypoxic insult are often overlapping in the young and adult brain. It is thought that decreased expression of cellular defence genes in the adult brain as opposed to the young one, renders the former less prepared and thus unable to withstand injury. Some evidence

exist which support this hypothesis. For example, Hif-1α levels are lower in the adult brain as opposed to the young one and might explain why mature cells have a decreased ability to withstand hypoxia. In a similar manner, cdk5 expression is reduced in the aged brain (150), suggesting that its ability to phosphorylate its substrates might also be decreased. It is clear that further research in this field is required to understand the complex regulation of signaling mechanisms that contribute to age-related neuronal susceptibility to injury.

Last but not least, we would like to address the influence of oxygen deprivation on cytoskeletal rearrangements, and progression of neurodegenerative diseases as a whole and particularly in relation to age. With our future studies we will provide further evidence for the crucial role of the neuronal cytoskeleton in progression of neurodegeneration and will inquire important information on the impact of environmental stimuli on neurodegenerative diseases.

6 ASTROCYTE RESPONSES TO INJURY: SIMULTAEOUS DEATH AND PROLIFERATION

6.1 **Objectives**

Susceptibility to oxygen deprivation varies greatly depending on the cell, astrocytes and endothelial cells being more capable to withstand hypoxic injury than neurons and oligodendrocytes (81). It is suggested that astrocytes can survive long periods of hypoxia as long as there is enough glucose for anaerobic glycolysis (239, 240). Astrocytes also have the additional role of supporting neuronal function and survival through their ability to take up glutamate and release of trophic factors (82-84). Following oxygen deprivation, astrocytes ensure their own survival as well as that of neighboring neurons by increasing glucose utilization and lactate release as a source of energy (241). Nevertheless, following a prolonged severe insult, astrocytic proliferation gives rise to a glial scar, a process that may interfere with neural repair and can thus impair brain recovery. Thus the astrocytic response to hypoxic/ischemic injury may depend to a large extent on the severity and also duration of an insult. Elucidating the time course of the astrocytic response to hypoxic/ischemic injury is important for preventing brain damage and pin pointing the time period when therapeutic intervention is possible.

The aim of this study was to elucidate the astrocytic response to mild and severe hypoxic/ischemic insults. In particular, we were interested in understanding the impact of oxygen and/or glucose deprivation on induction and stabilization of Hif-1α, a key regulator of hypoxia. In addition, with this study we aimed at directly addressing the impact of hypoxia and/or glucose deprivation on the vulnerability of

astrocytes. In light of this notion, we examined the effect of hypoxia/anoxia alone and in combination with glucose deprivation on astrocytic death.

6.2 Own Research

6.2.1 Anoxic but not hypoxic exposure induces Hif-1α

Most cells respond to changes in oxygen tension by initiating molecular pathways to combat oxidative stress. Hif-1 induction is one of the main mechanisms by which the cell adapts upon oxygen deprivation. The molecular mechanisms involved in the astrocytic hypoxic response and in particular the role of Hif-1 in these cells is not yet understood and was directly addressed in this study.

To this end, primary astrocytes were exposed to either hypoxic (1% O_2), or near anoxic (0.1% O_2) conditions with or without glucose for up to 48 hours. Our results show that hypoxia alone does not induce Hif-1α. Instead near anoxic conditions led to Hif-1α protein accumulation in a time dependent manner. This result was confirmed by EMSA, which showed Hif-1 DNA binding following anoxic exposure. Our findings agree with previous studies (72, 81) and emphasise that astrocytes are less vulnerable to injury than other brain cells such as neurons since mild oxygen deprivation was not sufficient to stabilise Hif-1. Nonetheless, glucose withdrawal alone induced low basal Hif-1α stabilisation and an ischemic insult (glucose/oxygen deprivation) led to an immediate accumulation of Hif-1, suggesting that in astrocytes glucose deprivation strongly induces Hif-1α stabilisation.

6.2.2 VEGF protein is induced by hypoxia in the absence of Hif-1α expression.

VEGF is a well described Hif-1α target gene. (113, 242). Notably, in astrocytes VEGF is present under normal physiological conditions but is further induced by

hypoxia leading to increased vascular permeability and glial cell proliferation (87) 56, 243). Hypoxia increased astrocytic cytoplasmic accumulation and basal release of VEGF in the absence of Hif-1α stabilization pointing to a Hif-1 independent regulatory mechanism following a mild hypoxic injury. In a similar manner, anoxia progressively increased VEGF release with a rapid increase observed only after 24h anoxia, suggesting that astrocytes produce similar VEGF amounts independently of the degree of hypoxia, but significantly increase VEGF production and secretion as a result of chronic exposure. Notably, oxygen/glucose deprivation led to a further increase in VEGF secretion, without affecting astrocytic cytoplasmic stores. Altogether our data suggest that increased severity of insult leads to an increase in VEGF secretion by astrocytes.

6.2.3 Cell death and proliferation occur in parallel following oxygen-glucose deprivation.

In vivo studies have shown that astrocytes respond to hypoxia/ischemia with increased proliferation. An interesting finding of this study is that different severities induced differential proliferation patterns *in vitro.* Enhanced proliferation was observed as a result of either oxygen (0.1% O_2) or glucose deprivation alone, which was further induced when oxygen and glucose were simultaneously withdrawn.

Interestingly, sole oxygen deprivation did not affect astrocytic survival at any time point. However, oxygen glucose deprivation significantly enhanced cell death after 6h, which is the time point when astrocytic proliferation was also increased.

Taken together our results suggest that astrocytes can tolerate oxygen deprivation for extended periods without induction of cell death pathways. At the same time cell

death is increased although proliferation levels were also high, indicating that there is a strong correlation between proliferation and cell death.

6.2.4 ATP levels are preserved despite oxygen and glucose withdrawal.

Cell survival under stress conditions requires constant ATP levels. Since astrocytes were able to survive oxygen deprivation for long periods of time we investigated whether and how cellular ATP levels were modulated by oxygen and/or glucose deprivation. Upon hypoxia astrocytes were able to maintain reduced but constant ATP levels, a situation which probably ensures astrocytic survival in hypoxic conditions. Interestingly, a dramatic drop in ATP levels was only observed in anoxic-glucose deprived cells, which were unable to recover from a 70% drop in ATP concentrations, underlining the increased resistance of astrocytes to reduced oxygen levels.

Thus astrocytes are able to maintain ATP levels and tolerate oxygen or glucose deprivation, but complete withdrawal of both leads to a dramatic drop on ATP levels.

6.3 **Discussion II**: Comparison of the astrocytes and neuronal hypoxic response.

One of the main characteristics of astrocytes, distinguishing them from other brain cells, is their ability to resist severe injury for a prolonged period of time (72, 81, 244). The results presented here are in line with previous studies showing that the hypoxic response differs remarkably from one cell to the other; astrocytes in particular are extremely resistant to oxygen deprivation, as opposed to neuronal cells. As shown in our work astrocytes are able to maintain ATP levels and thus survive oxygen deprivation for long period of times, as opposed to neurons which respond to hypoxia with a dramatic drop in ATP and an earlier onset of cell death. In astrocytes, oxygen in combination with glucose deprivation led to a dramatic drop of ATP levels followed by cell death. Our findings agree with other reports (244-246) showing that astrocytes can survive oxygen deprivation as long as glucose is available, partially due to their ability to maintain ATP levels better. Véga et al., (241) showed that decreased oxygen levels lead to an adaptive response that in astrocytes involves an increase in their glycolytic activity and lactate release. Such adaptation mechanisms might be employed by astrocytes to ensure their own survival but also that of neighbouring neurons, which is crucial to maintain brain function.

At the molecular level, oxygen sensing involves the induction of Hif-1. In astrocytes Hif-1α was stabilised only following severe oxygen deprivation whereas in neurons Hif-1α is stabilised immediately following hypoxic exposure. A number of questions arise from these studies about the role of Hif-1 in cell survival/death. Is the lack of Hif-1 accumulation directly responsible for the resistance of astrocytes to hypoxia? If so, would manipulation of Hif-1 stabilisation be beneficial for other cells, such as neurons? Notably, Halterman et al., (37) reported that delayed apoptotic neuronal

death is attenuated by expression of a dominant negative form of Hif-1α in an *in vitro* model of oxygen/glucose deprivation.

Of course Hif-1α also has positive functions since it induces a number of genes known to be involved in cytoprotection, amongst others, VEGF (26, 247) . We showed that neurons respond to hypoxia with an increase in VEGF mRNA levels in agreement with others (56, 85, 248). Neuronal VEGF induction contributes to neuronal survival and in a similar manner astrocytes respond to hypoxia by an increase in VEGF release. We suggest that an early increase in astrocytic VEGF release as a result of hypoxia maybe advantageous to the organism protecting neighbouring cells from injury through paracrine systems (249). Thus this mechanism may specifically provide additional support for neuronal survival in conditions of oxygen deprivation. On the other hand, the dramatic increase in VEGF release as a consequence of a more severe injury is probably negative and stimulates enhanced astrocytic proliferation, since it has been shown that in the CNS VEGF can promote astrocytic proliferation following injury (250, 251).

Another important determinant of brain injury is age (74, 252, 253). Mature neurons responded to hypoxia with a dramatic drop in ATP levels coinciding with an increased cell death. Although not addressed in this study, similar differences have been documented in immature versus mature astrocytes *in vitro.* Mature astrocytes are less able to survive profound decreases in ATP levels and are more vulnerable to oxygen/glucose deprivation compared to immature astrocytes (252, 254) In this regard it has been suggested that mature brain cells, with a high rate of oxidative metabolism and thus higher energy expenditure, utilise their energy stores more rapidly (75, 255). How age would affect astrocytic function in relation to neuronal support is an interesting question and deserves further investigation. However we can

speculate that mature astrocytes, being more vulnerable to injury, will also have a reduced ability to sustain neuronal survival. Notably, astrocytes have now been implicated in the pathogenesis of age-related neurodegenerative disorders, including Alzheimer's (256, 257). It is thought that the presence of glial scars, as a result of astrocytic proliferation, in Alzheimer's brains, directly contribute to disruption of synaptic function and neuronal loss (258). To date, scientists have the task to better understand the intrinsic interactions between astrocytes and neurons and how these are affected as a result of injury and aging.

6.4 Conclusions II and Outlook II

The main focus of this thesis work was to characterise cellular and molecular mechanisms involved in the neuronal response to oxygen deprivation. At the same time we were interested in understanding how the astrocytic response differs from that of neurons and contributes to the enhanced resistance of astrocytes to hypoxia.

Evaluation of hypoxia in brain *in vivo* is complex, since systemic hypoxia leads to cardiac failure and subsequent cerebral ischemia (259). Furthermore, and as shown also in this study, although common molecular mechanisms do exist the cellular and molecular changes in any given cell can be diverse. Therefore *in vitro* cell cultures are an essential simplified model system, which give us the possibility to evaluate cell morphology, function and identification of signalling pathways in the response of a particular cell type to injury. Nevertheless, it is crucial to increase our knowledge on the physiological interactions between neurons and astrocytes but also to understand how these interactions are affected as a result of injury, information that can be provided by *in vitro* co-culture systems. Such knowledge will partially contribute to our understanding of the complexity of the nervous system and can be subsequently applied to *in vivo* models to better understand the pathophysiology of diseases.

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7 References

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8 Manuscripts

Cdk5 modulates Hif-1 in hypoxic neurons

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ABSTRACT

The cyclin dependent kinase 5 (cdk5)/p35 complex is essential for regulation of the cytoskeleton and cell survival during development and in models of neuronal excitotoxicity. Dysregulation of cdk5, by cleavage of its neuronal specific activators p35 and p39, has been implicated in various neurodegenerative disorders such as Alzheimers disease (AD) but targets of the complex that regulate neuronal survival physiologically and/or during pathogenesis are largely unknown. Since hypoxia also characterizes many (neuro)pathological states and also causes cytoskeletal disruption, we investigated a possible contribution of the cdk5/p35 complex to the neuronal hypoxic response. In primary cortical neurons hypoxia disrupted the F-actin cytoskeleton culminating in cell death induction as well as transiently increased cdk5 and p35 transcription. Abundance of p35 protein also increased during hypoxia resulting in increased cdk5 activity. Importantly, cdk5-mediated signaling was crucial for activation of hypoxic responsive pathways since cdk5 inhibition by roscovitine (20μM) abrogated Hif-1α stabilisation and hindered Hif-1α target gene expression.

Taken together our results show that the cdk5/p35 complex is regulated by hypoxia, plays a pertinent role in modulation of Hif-1α stabilisation and impacts Hif-1-induced signaling pathways. This study highlights a new hypoxia-mediated signaling pathway and implicates the cytoskeleton as a potential regulator of Hif-1α.

Key words: cdk5/p35, oxygen deprivation, cytoskeleton, cell death, erythropoietin

INTRODUCTION

Efficient oxygen delivery to brain tissues is essential for cell survival and normal brain function. Hypoxia, an important stimulus characterising a wide variety of physiological events, also contributes significantly to progression of pathogenesis and widespread diseases including cancer, stroke and neurodegenerative disorders. Although a hypoxic stimulus is known to induce signal transduction pathways that activate transcriptional factors ultimately determining cell fate, limited data is available on very early hypoxia-induced signaling events that lead to cell death.

An important role for the cytoskeleton in mediation of signal transduction after a stressful stimulus is becoming increasingly apparent. Disruption of the cytoskeletal network by hypoxia has been reported in various tissues such as the vascular system, brain, kidney and lung (Bouvry et al., 2006; Lee et al., 2001; Roberts-Lewis and Siman, 1993). Oxygen deprivation in the brain causes CNS neurons to undergo morphological changes, including process retraction and loss of shape, partially attributable to depolymerisation of actin filaments (Friedman et al., 1998).

The serine/threonine protein kinase cdk5 together with its neuronal specific activators, p35 and p39, is an important regulator of the neuronal cytoskeleton (Smith, 2003). Cdk5 is a multifunctional kinase involved in a variety of normal neuronal functions through phosphorylation of a wide variety of proteins (Maccioni et al., 2001). Cdk5 localises to the growing tips of developing neurites and is able to regulate the dynamics of microtubules and actin microfilaments (Namgung et al., 2004; Ohshima et al., 1996). Cdk5 also makes an important contribution to neuronal survival. Li et al., (Li et al., 2002) reported a key role for the kinase in promoting neuronal survival via negative regulation

of the pro-apoptotic JNK3 pathway in UV-exposed neurons and a recent study by Wang et al. (Wang et al., 2006) suggested that cdk5 activity is required to protect hippocampal neurons from serum deprivation. Conversely, other evidence suggests that abnormal cdk5 activation is neurotoxic leading to apoptosis under both physiological and pathological conditions (Shelton et al., 2004). Indeed deregulation of cdk5 activity has been reported in a number of neurodegenerative disorders including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Lau et al., 2002; Nguyen et al., 2001). Under neurotoxic conditions, proteolytic cleavage of p35 by calpain results in the generation of a more stable fragment, p25, resulting in mislocalisation of the kinase, deregulation of its activity, disruption of the cytoskeleton and eventually neuronal death (Kusakawa et al., 2000; Patrick et al., 1999). Increased p35 levels and cdk5 activity in neurons was also reported after middle cerebral artery occlusion (Hayashi et al., 1999) as well as in human stroke tissue (Mitsios et al., 2007) implicating a role for the complex in the neuronal response to ischemic injury. Thus cdk5 regulation is complex and has distinct functions depending on the stress stimulus and cell type.

At the molecular level, a large percentage of hypoxic/ischemic responses are controlled by the transcription factor hypoxia-inducible factor-1 (Hif-1) (Fandrey et al., 2006; Hopfl et al., 2003). Hif-1 is a heterodimer composed of an oxygen-dependent Hif-1α subunit and a constitutively expressed Hif-1β subunit. Under normoxic conditions Hif-1α is hydroxylated, ubiquinated and rapidly degraded by proteasome 26S. Upon hypoxia, stabilised Hif-1α translocates to the nucleus and dimerises with Hif-1β ultimately leading to increased transcription of a number of gene products. Among others (Semenza, 1999), Hif-1 regulates the transcription of vascular endothelial growth factor (VEGF) and

erythropoietin (Epo), which have known neuroprotective functions (Gassmann et al., 2003; Ogunshola et al., 2002). Despite the fact that new advances currently point to the involvement of the cytoskeleton in Hif-1 regulation (Escuin et al., 2005; Shen et al., 2007) the mechanisms that couple the cytoskeleton to Hif-1 are still to be identified.

To explore the possibility that hypoxia may directly regulate the activity of the cdk5/p35 complex, and whether cdk5 itself can contribute to the neuronal response to oxygen deprivation, we employed an *in vitro* model of primary mouse neurons. We show that hypoxia indeed regulates the cdk5/p35 complex, which in turn modifies the neuronal hypoxic response via modulation of Hif-1 mediated pathways.

MATERIALS AND METHODS

Primary neuronal cultures. Primary neuronal cultures were obtained from the cerebral cortex of C57Bl/6J mice (gestational stage E14) (Ogunshola et al., 2002). Dissected cortices were dissociated in Hank's buffered salt solution (HBSS) containing trypsin and DNase I for 5min at 37°C. Neurons were seeded in poly-L-lysine coated petri dishes (3 X 10^6 cells per 100mm dish) in glucose-containing DMEM medium containing: B27 supplement (1X), albumax (0.25g/ml), sodium pyruvate (1%) and 100U/ml streptomycin-penicillin, (GIBCO, Invitrogen, AG, Switzerland). Neurons were maintained in culture for 6 days (6DIV) at normal atmosphere (21%O_2) in a humidified incubator at 37°C.

Hypoxic experiments and inhibitor treatment. Hypoxic experiments were performed in a purpose-built glove-box chamber (In vivo 400, RUSKINN Technologies, Guiseley, UK) maintained at 37°C with 5% CO_2. Neurons were exposed to 1% O_2 for different periods of time and cell harvesting was performed within the chamber (i.e. without reoxygenation

of samples). Normoxic control cultures were maintained at 21% O_2. Cdk5 activity was inhibited by direct application of 20µM roscovitine (Calbiochem, Lucerne, Switzerland) to the neuronal cultures immediately prior to hypoxic exposure.

ATP measurements. An ATP bioluminescent assay kit (Sigma, Buchs, Switzerland) was used according to manufacturer's instructions. Following hypoxia cells were lysed in lysis buffer (150mM NaCl, 50mM Tris, 1% Triton X-100, 1% NP-40) supplemented with protease inhibitors (Roche, Basel, Switzerland) for 10 min followed by 10 min centrifugation at 16'000 rcf. The supernatant was mixed with 5% TCA (1:1 v/v) to block residual ATPase activity and luminescence was measured using a Berthold luminometer (Detection systems GmbH, Pforzheim, Germany). ATP amounts were determined from a standard curve and total amount of ATP was normalised to total protein concentrations.

Assessment of cell death. Neuronal cell death was quantitatively assessed using a colorimetric assay for detection of lactate dehydrogenase (LDH) release according to the manufacturer's instructions (Roche, Penzberg, Germany).

Real-Time PCR (RT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen AG, Switzerland) according to the manufacturer instructions. 2µg RNA was used for cDNA synthesis using the QuantiTect Reverse Transcription kit (Qiagen AG, Hombrechtikon, Switzerland). Analysis was performed with 1µl of the final cDNA mix using mouse-specific Taq-Man-based gene expression assays (Applied Biosystems). The following assays were used in an ABI 7500 RT-PCR thermocycler (Applied Biosystems,

Foster City, CA): 28sRNA (catalog no.: Mm00508318_m1), p35 (catalog no.: Mm00438148_s1), cdk5 (catalog no.: Mm00432437_m1), Epo (catalog no.: Mm00433126_m1), VEGF (catalog no.: Mm00437304_m1). Data were normalised to 28sRNA and normoxic controls. All reactions were run in duplicate and at least three independent experiments were performed.

Western Blot. Cells were lysed in lysis buffer (150mM NaCl, 50mM Tris, 1% Triton X-100, 1% NP-40) for 10min and centrifuged at 16'000 rcf for 10 min (4°C) and the supernatant collected. 75µg of protein were loaded on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated with anti-p35 (C-19, 1:500, Santa Cruz, CA, USA), anti-cdk5 (C-8, 1:250, Santa Cruz, CA, USA), anti Hif-1α (NB 100-479, 1:1000, NOVUS, Littleton, USA) and anti-Tuj1 (SIG 3840, 1:7000, COVANCE, Berkeley, California). Following washes, membranes were incubated with a secondary HRP-conjugated antibody for 1 hour at room temperature. All blots were normalised to Tuj-1 and normoxic controls and at least 3 independent experiments were performed. Results were quantified by densitometry using BioRad Quantity One software (BioRad laboratories AG, Switzerland).

Cdk5 kinase assay. 50µg of whole cell protein were incubated with 2µg of anti-cdk5 (C-8, Santa Cruz, CA, USA) for 2h and precipitated with protein A-sepharose beads (Amersham, Buckinghamshire, UK) for 1h at 4°C. Immunoprecipitated complexes were incubated with 10 µg of histone H1 and 1µCi of ^{32}P in kinase buffer (10mM Tris, 1mM DTT, 2mM EGTA, 10mM $MgCl_2$, 20mM ATP) for 30min at room temperature. The

reaction was stopped with the addition of Laemmli buffer and samples were denaturated at 95°C for 5 min, then loaded on a 15% SDS-PAGE gel. After running, the gel was dried in a slab gel dryer system (Witec AG, Littau) at 80°C for 2 hours then exposed to a phosphoscreen and visualised using a BioRad Molecular Imager FX.

*Immunocytochemistry***.** Neurons were plated on coverslips in 24 well plates (70,000 cells per well), grown for 6 days and then subjected to hypoxia. Cultures were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 5min at room temperature then permeabilised in 0.1%Triton X-100 for 1min and blocked with 5% normal goat. Cells were then incubated with the primary antibodies anti-p35 (C19, 1:500, Santa Cruz, CA) or anti-cdk5 (C8, 1:500, Santa Cruz, CA) overnight at 4°C. Secondary antibody to p35 was Cy3 (Jackson ImmunoResearch, West Grove, PA), and to cdk5 was AlexaFluor 488 (Molecular Probes, Leiden, Netherlands). Staining for F-actin was performed with phalloidin-TRITC (1:500, SIGMA, Missouri, USA) for 40min at room temperature. Slides were viewed and analysed using an Axiovert inverted fluorescent Microscope (Zeiss, Germany). Quantification analysis was performed using MCID analysis 7.0 software (Imaging Research Inc.). Fluorescent phalloidin intensity was measured in 12 individual fields of 3 independent experiments. Histograms were subsequently obtained using the grey values (on a scale of 0-255 for black to white) obtained for each images. Median fluorescence density for each field of every time point was then graphed.

Statistical analysis Graphs and analyses were performed using GraphPad Prism software. Statistical comparison among groups was made using one-way and two-way ANOVA

tests. A value of $p<0.05$ was considered significant. All results are expressed as mean ± standard deviation.

RESULTS

Hypoxia decreases ATP levels and neuronal survival

Reduction of cellular ATP levels is an early event in response to periods of energy deprivation. Fig.1A shows changes in intracellular ATP levels in neurons under hypoxic conditions. Within 2h, ATP levels decreased by approximately 70% compared to normoxic controls then stabilised till 6h ($p <0.01$). By 24h ATP levels had decreased by approximately 85% ($p <0.01$) indicating exceedingly low energy levels. Since both reduced ATP and hypoxic exposure have been linked to cell death we measured LDH leakage, an established index of neuronal death (Fig. 1B). Surprisingly, despite the dramatic fall of ATP levels, hypoxia only induced significant neuronal death after 6h (6.610%±0.57; $p<0.05$) with an additional increase to 10.61%± 3.84 observed at 24h ($p<0.01$). Thus cortical neurons can survive 1% O_2 for extended periods of time.

Hypoxia disrupts the neuronal F-actin cytoskeleton

Early breakdown of actin microfilaments is a prerequisite for cell-shape alterations and cell death following a stressful stimulus (Kuhne et al., 1993), thus we investigated the effect of hypoxia on actin microfilament organisation in primary neuronal cultures (Fig. 1C; a-d). Immunocytochemistry with TRITC-labelled phalloidin showed that normoxic controls exhibit a well defined F-actin network with homogeneous distribution of phalloidin staining in neuronal dendrites (Fig. 1C, a). In contrast, hypoxic exposure of

neurons for 2h resulted in disruption of the actin network with clear retraction of filaments (Fig.1C, b, arrow). The severity of disruption was time dependent and characterised by further retraction of the F-actin fibers by 4h and 6h (Fig. 1C, c-d). Quantitative analysis of the area of phalloidin staining revealed a time-dependent increase in peak counts close to zero on the grey scale (i.e. less area stained) during hypoxia as shown by the representative histogram (Fig. 1D). The net result was a significant decrease in median fluorescence intensity observed following 4h and 6h exposure (Fig.1E) confirming a hypoxic-induced decrease in the area of F-actin staining. Thus, hypoxia-induced alterations in the neuronal F-actin cytoskeleton correlate with decreased ATP levels and increased cell death.

p35 is reversibly dephosphorylated and protein levels increase in response to hypoxia

The effect of hypoxia on the cdk5/p35 complex, an important regulator of the neuronal actin cytoskeleton, was assessed in primary cortical neuronal cultures. Hypoxia rapidly upregulated p35 mRNA levels with a peak (60 fold ± 5.20) observed at 2h (Fig. 2A, $p < 0.01$). This increase was transient, and mRNA levels returned to baseline by 4h. Increased p35 protein levels paralleled the increase in mRNA (Fig. 2B). Quantification shows p35 protein levels increased 1.8 fold (± 0.55) after 2h and remained elevated till 4h but were then significantly downregulated. A change in the electrophoretic mobility of p35 after 2h of hypoxia was also observed suggesting protein dephosphorylation (Fig. 2B). Hypoxia-induced dephosphorylation was rapid, occurring within 30 min (Fig. 2C) but reversible after 4h reoxygenation as indicated by the reappearance of the higher

molecular weight band. Thus p35 phosphorylation is linked to oxygen availability. Notably, hypoxic exposure did not lead to production of p25, the p35 breakdown product. Using immunocytochemistry we localised p35 throughout the neuronal soma and neurites (Fig. 2D) however hypoxia did not affect p35 cellular distribution.

Altogether, our results show that oxygen deprivation modulates p35 both at the transcriptional and translational level. However, despite the hypoxic insult, p35 cleavage was not observed suggesting that calpain pathways are not activated.

Cdk5 activity is upregulated by hypoxia

Similar to p35, peak induction of cdk5 mRNA occurred at 2h of hypoxia (25.92 ± 7.87; $p < 0.01$; Fig. 3A) and stayed elevated at 4h (11.75 ± 1.6) before returning to baseline by 6h. Surprisingly, cdk5 protein levels were not affected by hypoxia, remaining constant at all time points (Fig. 3B). Using a phosphorylation-specific antibody that targets the Ser159 residue of cdk5, a marker of protein activation (Sharma et al., 1998), we observed a transient increase in phosphorylation after 2h and 4h of hypoxia indicating increased cdk5 activity (Fig. 3C). To confirm this observation we performed *in vitro* kinase assays (Fig. 3D) and again cdk5 activity increased at 2h and was elevated further by 4h. This was consistent with both the phosphorylation state of cdk5 and increased p35 levels, underlying the fact that cdk5 activity is regulated by p35. Cdk5 activity was only transient and returned to basal levels but remained detectable at 24h even in the absence of p35 suggesting that other mechanisms may compensate for loss of p35.

Immunostaining analysis (Fig. 3E) showed that under normoxic conditions cdk5 is mainly cytoplasmic but after 4h of hypoxia, when activity of cdk5 is maximal,

localisation of the protein to the neuronal soma is clearly visible (Fig. 3E, arrows). The mislocalisation of cdk5 to the neuronal soma suggests possible interaction of the kinase with different effectors.

Cdk5 is necessary for Hif-1α stabilisation

The involvement of cytoskeletal regulatory proteins in Hif-1 modulation has been described in recent reports (Shen et al., 2007; Turcotte et al., 2003). Since cdk5 regulates the F-actin cytoskeleton and hypoxia induced cdk5 activity, we investigated whether cdk5 itself regulates Hif-1α. As expected hypoxia caused a time-dependent neuronal accumulation of Hif-1α (Fig.4A). Application of the cdk5-specific pharmacological inhibitor roscovitine (20μM) prior to hypoxic exposure attenuated temporal accumulation of Hif-1α during hypoxia (Fig. 4B). Normalisation confirmed a 50% downregulation of Hif-1α by 6h (Fig. 4C, $p < 0.01$).

To elucidate whether the observed decrease of Hif-1α induction by cdk5 inhibition was transient, neurons were treated with roscovitine and exposed to prolonged hypoxia. Remarkably, although Hif-1α was still detectable in control cultures, roscovitine treatment completely abolished Hif-1α accumulation (Fig. 4D) demonstrating that cdk5 activity is essential to Hif-1α stabilization.

Cdk5 inhibition downregulates Epo but not VEGF mRNA levels

Cdk5 inhibition attenuated Hif-1α accumulation suggesting an impact on Hif-1-mediated signaling pathways. Thus the effect of cdk5 inhibition on Hif-1 inducible neuroprotective pathways was investigated. For this purpose Epo and VEGF mRNA levels were assessed

by RT-PCR. As expected, hypoxia upregulated Epo and VEGF mRNA levels in a time-dependent manner in untreated cells (Fig. 5A, B). Application of roscovitine completely abolished Epo mRNA levels ($P < 0.001$) by 24h drug application. Surprisingly, in contrast VEGF mRNA levels were not significantly affected by roscovitine treatment at any time point. Thus cdk5 seemingly has differential effects on Hif-1α target genes involved in neuroprotection following a hypoxic insult.

Cdk5 inhibition leads to increased cell death only during chronic hypoxia

Involvement in regulation of Hif-1α-inducible pathways suggested a role for cdk5 in hypoxic neuronal survival. In our cultures hypoxia alone did not induce cell death until 6h exposure which then subsequently increased by 15.74% ± 1.71 at 36h and 26.54% ± 1.58 at 48h (Fig. 6). Roscovitine application did not detectably alter neuronal survival in normoxic cultures or cells exposed to up to 24h hypoxia compared to controls. However during extended exposure drug treatment significantly increased cell death (27.46% ± 8.43 and 38.27% ± 3.69 after 36h and 48h respectively) compared with hypoxia alone. *Exposure of normoxic neurons to roscovitine treatment for 48h showed non-toxicity of the drug by absence of cell death.* Thus cdk5 inhibition renders neurons more vulnerable to prolonged hypoxic insult.

DISCUSSION

The cytoskeleton is a highly dynamic structure that determines both structural and functional integrity of a cell and is known to be crucial in early neuronal responses to low levels of oxygen. Accordingly, cytoskeletal alterations have been documented in a

number of pathogenic diseases and neurodegenerative disorders characterised by oxygen deprivation (Magin et al., 2004). The cdk5/p35 complex also seems to play a crucial role in regulating neuronal cytoskeletal dynamics during both development and disease (Smith, 2003). This study provides new evidence that cdk5, and its neuronal activator p35, play a direct role in shaping the neuronal hypoxic response.

The first evidence of a role for the cdk5/p35 complex in neuronal responses to oxygen deprivation came from research in *Drosophila* (Ma and Haddad, 1999). Since then a number of studies have reported upregulation of the complex in models of ischemia both *in vivo* and *in vitro* (Hayashi et al., 1999; Mitsios et al., 2007; Rashidian et al., 2005; Strocchi et al., 2003). However how oxygen deprivation alone specifically regulates the complex in mammalian cells has not been demonstrated. Our results show that hypoxia alone induces cytoskeletal rearrangement and distinctly modulates both components of the complex, in agreement with another study showing increased activation of cdk5 in neuroblastoma cells subjected to chemical oxidative stress (Strocchi et al., 2003). Thus hypoxia and ischemia both cause significant alterations in cdk5 that could have consequences for neuronal function and survival. The localisation of cdk5 is also an important feature of neurotoxicity that could determine neuronal fate (O'Hare et al., 2005). *In vitro*, both cdk5 and p35 have been localised to the soma and along neurites (Nikolic et al., 1998; Qu et al., 2007) as well as the nucleus (O'Hare et al., 2005). Hypoxia caused redistribution of cdk5 from over the entire cell to mainly the neuronal soma, suggesting an altered function for the kinase and possible interaction with aberrant proteins. Notably, it has been shown that nuclear translocation of cdk5 can result in modulation of transcription factors that are involved in cell death pathways (O'Hare et al.,

2005).

Nowadays it is becoming evident that binding of cdk5 to p25 is required to render cdk5 a pro-death protein. Although prolonged activation and mislocalisation of the protein as a result of binding to p25 leads to cytoskeletal damage and apoptosis, an event linked to Alzheimer's disease (Patrick et al., 1999), on the other hand p35 may also regulate, and be required for, cdk5 pro-survival signaling. Although hypoxia did not affect p35 localisation, a change in the electrophoretic mobility of the protein was noted. Previous reports have linked p35 dephosphorylation to decreased ATP levels (Saito et al., 2003) an observation also observed in our study, however reoxygenation reversed this dephosphorylation. The importance of this change and its reversibility is unclear. It is believed that phosphorylated p35 is more resistant to calpain cleavage as opposed to unphosphorylated p35 which is easily cleaved to p25 when calpain is activated (Saito et al., 2003). However hypoxic-induced dephosphorylation of p35 did not lead to p25 formation in our study, indicating that calpain pathways were not initiated. Thus the role of p35 during hypoxic exposure remains intriguing and deserves further investigation. Despite this significant cell death after prolonged periods of hypoxia, and exacerbation in the presence of roscovitine, provides strong evidence that cdk5 activity is required for neuronal survival following hypoxic insult. Thus it appears that increased cdk5 activity in the absence of p35 cleavage probably confers a neuroprotective role in agreement with other reports (Li et al., 2002; Vartiainen et al., 2002).

Although we have not identified the mechanism of alterations in complex activity, disruption of the F-actin network during hypoxia may play a significant role. Evidence shows that cytochalasin D leads to a drastic re-organisation of the F-actin cytoskeleton

and an increase in cdk5 activity in COS7 cells transfected with p39, another cdk5 activator (Humbert et al., 2000). Cytochalasin D-induced redistribution of p39 protein to F-actin clusters further suggested that availability of the activator is the limiting factor determining cdk5 activity. Lee et al. (Lee et al., 1996) showed that the cdk5/p35 complex associates with a kinase inhibitor protein and this macromolecular complex can be destabilised by certain stimuli, leading to an increase in the availability of the cdk5 activators ultimately increasing kinase activity. It seems likely that hypoxia, through its disruptive effect on the actin cytoskeleton, is such a stimulus.

Cellular responses to reduced oxygenation are mainly controlled by the transcription factor Hif-1 (Fandrey et al., 2006; Hopfl et al., 2003) and involvement of the cytoskeleton in Hif-1 modulation has been a recent matter of discussion. Indeed microtubule-targeting agents down-regulate Hif-1 in cancer cell lines exposed to hypoxia (Escuin et al., 2005) and a role for the cytoskeleton-regulating protein, Rac1, in the modulation of Hif-1α in a carcinoma cell line was recently reported (Gorlach et al., 2003; Hirota et al., 2004). In the present study pharmacological inhibition of cdk5 in hypoxic cortical neurons significantly attenuated Hif-1α expression. These experiments for the first time provide direct evidence that cdk5 is involved in regulation of Hif-1. Notably the inhibition of Hif-1α accumulation was time-dependent showing that cdk5 is essential to induction of crucial adaptive pathways particularly during prolonged hypoxia. The mechanism behind this inhibition is still unclear. Notably, cdk5 is a serine/threonine kinase and application of a general serine/threonine kinase inhibitor was shown to significantly inhibit Hif-1 hypoxic induction (Wang et al., 1995). Importantly, we have identified a putative cdk5 phosphorylation site within the Hif-1α protein sequence suggesting that cdk5 could

potentially phosphorylate Hif-1α directly and affect its accumulation. However an indirect effect of cdk5 cannot be ruled out and additional studies are now being performed to answer this question.

Disruption of HIF-1α function must have significant consequences on cell survival since induction of target gene expression, signaling and subsequent hypoxic adaptation are also abrogated. Several *in vitro* models show that neurons exposed to hypoxia significantly upregulate Epo (Genc et al., 2004) and VEGF mRNA and protein levels (Jin et al., 2000; Ogunshola et al., 2002). In addition, pretreatment of neurons with Epo and VEGF prevents neuronal death in *in vitro* models of hypoxia and reduces ischemic damage in various animal models of stroke (Jin et al., 2000; Liu et al., 2006). Surprisingly, although Epo gene expression was prevented by cdk5 inhibition, VEGF mRNA levels were unaffected. Thus cdk5 modulates Hif-1 signaling pathways but, intriguingly, has differential effects on Hif-1 downstream targets. Indeed our results appear to be in line with Chavez et al., (Chavez et al., 2006) who reported that Hif-1 binds preferentially to the Epo promoter in neurons. Ultimately our data point to the importance of Epo signaling in neuroprotection, since its inhibition by roscovitine also correlates with increased cell death in hypoxia. Considering the importance of the cdk5/p35 complex in neurodegenerative disorders, and the recent demonstration that hypoxia is an ongoing state in many of these diseases, it is now crucial to explore whether the identified mechanism is universal or restricted to neurons in an early developmental stage.

In conclusion this study shows that oxygen deprivation regulates the p35/cdk5 complex, and for the first time cdk5 is identified as a regulator of Hif-1α accumulation and Epo-mediated signaling. The implications of these findings are of significant importance for

understanding the contribution of hypoxia-driven cytoskeletal regulation to neuronal survival and ultimately progression of neurodegenerative diseases.

FIGURE LEGENDS

Fig. 1. Hypoxia reduces neuronal survival and disrupts cytoskeletal structure.
A) Intracellular ATP levels are significantly reduced during hypoxic exposure. A 70% reduction is observed already after 2h that further decreases at 24h. B) LDH assay shows hypoxia modestly increases neuronal cell death. A small but significant increase in LDH release is observed only after 6h of hypoxia. C) The neuronal cytoskeleton is severely disrupted by hypoxia. Cortical neurons were double stained for F-actin (TRITC-phalloidin) and nuclei (DAPI). Representative photomicrographs show time-dependent retraction of F-actin fibers following hypoxic exposure (b-d) compared to control cultures (a). D & E) Representative histograms show quantitative analysis of phalloidin staining intensity in both control and hypoxic neurons. Increased narrow distribution and higher peaks is observed with increased hypoxic exposure (D), indicating decreased distribution of F-actin staining. E) Graph shows significant decrease in median fluorescence intensity (i.e. reduced area of staining) as a result of hypoxic exposure further confirming decreased F-actin levels with hypoxic exposure. * denotes $p < 0.05$, ** denotes $p < 0.01$.

Fig. 2. p35 is regulated by hypoxia at the transcriptional and translational level.
A) Real-time PCR demonstrates a large but transient increase in p35 mRNA expression after 2h of hypoxia ($p < 0.01$). B) Representative Western blot for p35 shows protein levels are initially increased by hypoxic exposure but later down regulated. Quantification of Western blots shows a 1.8 fold ± 0.5 increase in p35 expression after 2h of hypoxia ($p < 0.01$). Values are representative of at least 3 independent experiments; all blots are normalised to Tuj-1. A change in p35 mobility after 2h hypoxic exposure is also observed. Notably, p25 is not detected. C) Western blot analysis shows that the electrophoretic mobility of p35 is altered already after 30 min of hypoxia. Reoxygenation for 4h demonstrates that the phosphorylation state of p35 is reversible. D) p35 immunohistochemistry shows protein localisation is unaltered by hypoxic exposure. In all cases p35 localised to the neuronal soma and dendrites both in normoxia and hypoxia. Nuclei were co-stained with the nuclear marker DAPI. Scale bar = 20µm.

Fig. 3. Activity of cdk5 is induced by hypoxia.

A) RT-PCR shows that cdk5 mRNA levels are significantly but transiently induced by hypoxia. ** denotes a P value of < 0.01. B) Western blot analysis shows that cdk5 protein levels are not affected by hypoxia. C) Western blot analysis indicates a transient increase in phosphorylated cdk5 following 2h and 4h of hypoxia. D) Representative kinase activity assay (using Histone H1) shows transient upregulation of cdk5 activity by hypoxia. Upregulation occurred at 2h and 4h before returning to baseline. E) Neuronal localization of cdk5 is altered by hypoxic exposure. Representative photomicrographs of staining show the translocation of cdk5 to the neuronal soma after 4h of hypoxia. Nuclei were co-stained with the nuclear marker DAPI. Scale bars = 20μm.

Fig. 4. Cdk5 inhibition attenuates Hif-1α hypoxic induction.

A) Hypoxia induces time-dependent induction of Hif-1α in neurons. Hif-1α is induced following 2h exposure to hypoxia and increases up to 24h. B) Representative Western blot of Hif-1α after hypoxic exposure shows roscovitine (20μM) inhibits Hif-1α expression. C) Quantification shows significant downregulation (50%) of Hif-1α hypoxic induction by 6h ($p < 0.01$). D) Representative Western Blot of Hif-1α induction during prolonged hypoxia after cdk5 inhibition. Hif-1α expression is completely abolished after 24h of hypoxia in the presence of roscovitine.

Fig. 5. Cdk5 inhibition abrogates Epo but not VEGF mRNA levels.

The effect of roscovitine treatment on neuronal hypoxic induction of Epo and VEGF mRNA levels was assessed by RT-PCR. A) Roscovitine attenuates hypoxic-induced Epo mRNA expression after 24h. B) Roscovitine does not alter hypoxic induction of VEGF mRNA levels. All values are normalised to 28sRNA and normoxic controls. ($p < 0.001$).

Fig. 6. Neuronal survival during prolonged hypoxia is reduced by cdk5 inhibition.

LDH assay was performed on media from roscovitine-treated neurons exposed to hypoxia. Hypoxic exposure alone caused a time-dependent increase in cell death. Roscovitine treatment significantly enhanced cell death in the hypoxic, but not normoxic, cultures after prolonged hypoxic exposure (** denotes a $p < 0.01$).

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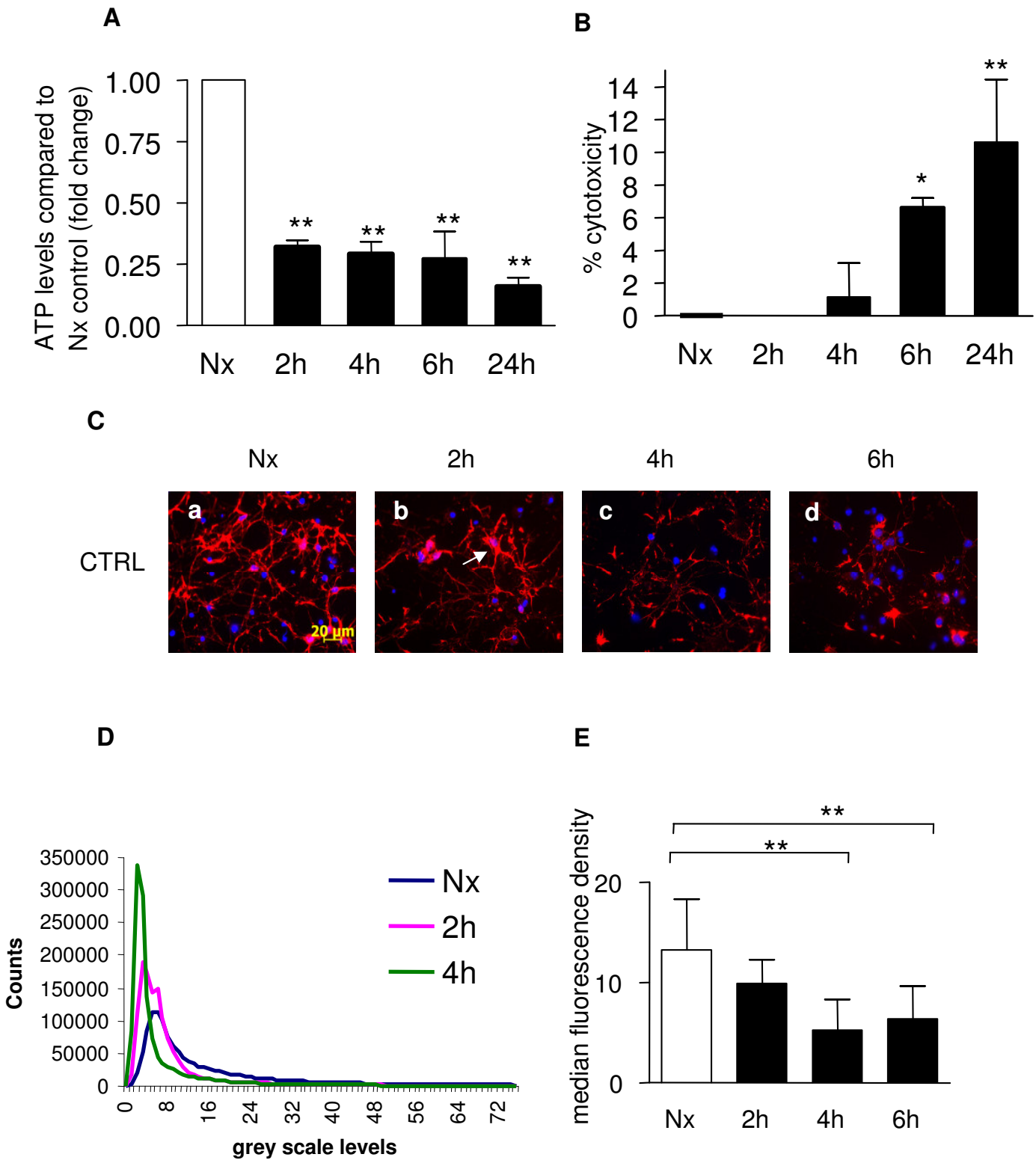
Fig. 1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ATP levels compared to Nx control (fold change)
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0.25
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12
10
8
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4
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Nx
2h
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Counts
350000
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250000
200000
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8
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72
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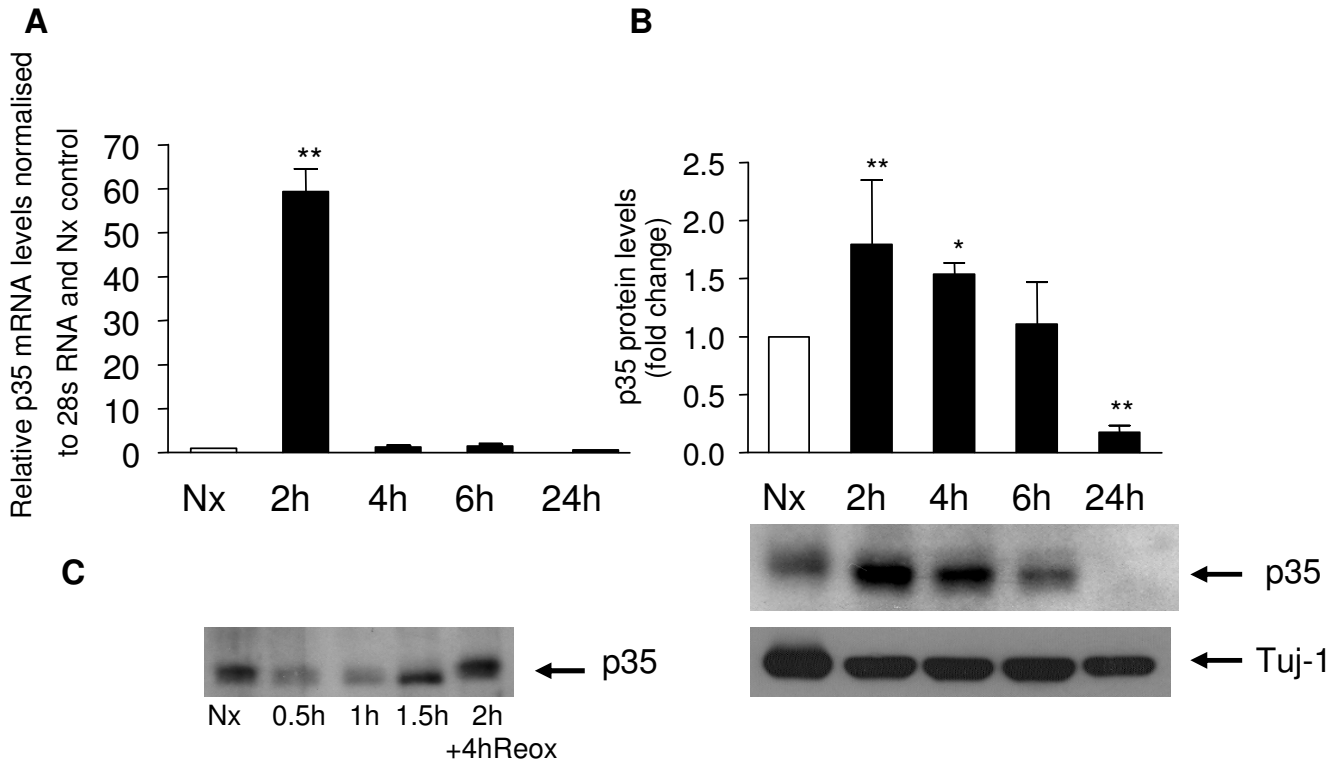
Fig. 2


A
Relative p35 mRNA levels normalised to 28s RNA and Nx control
70
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50
40
30
20
10
0
**
Nx
2h
4h
6h
24h
B
p35 protein levels (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
**
*
**
Nx
2h
4h
6h
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C
p35
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1.5h
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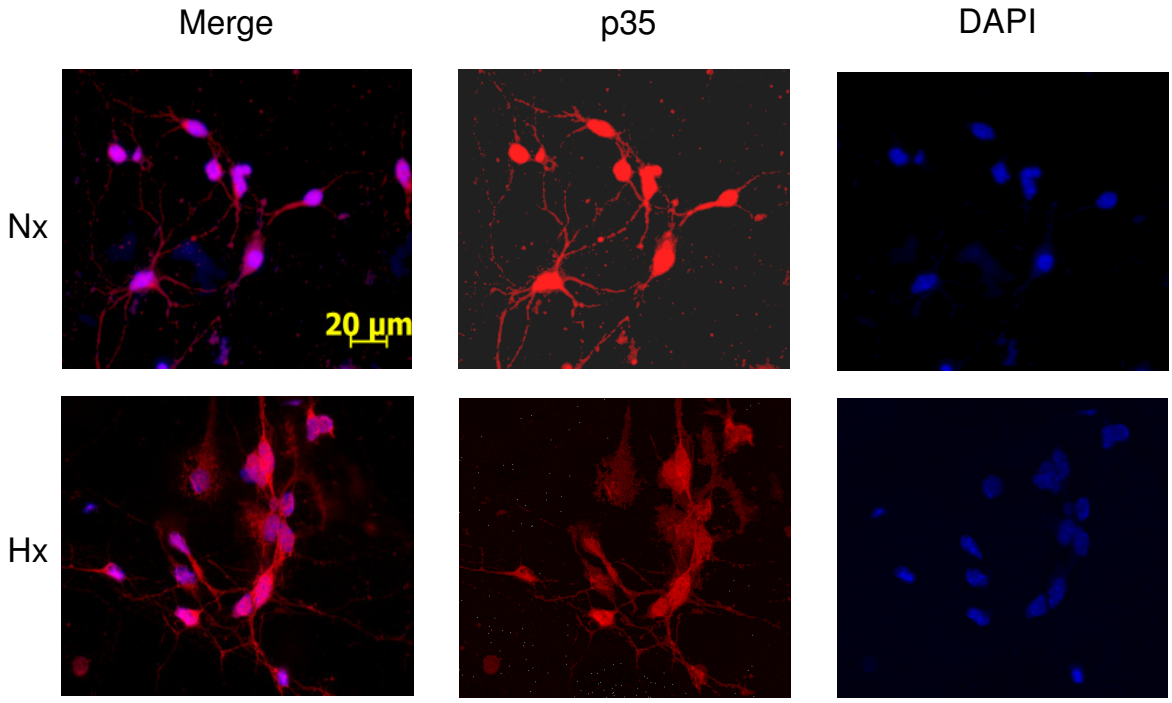
+4hReox
p35
Tuj-1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p35
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Nx
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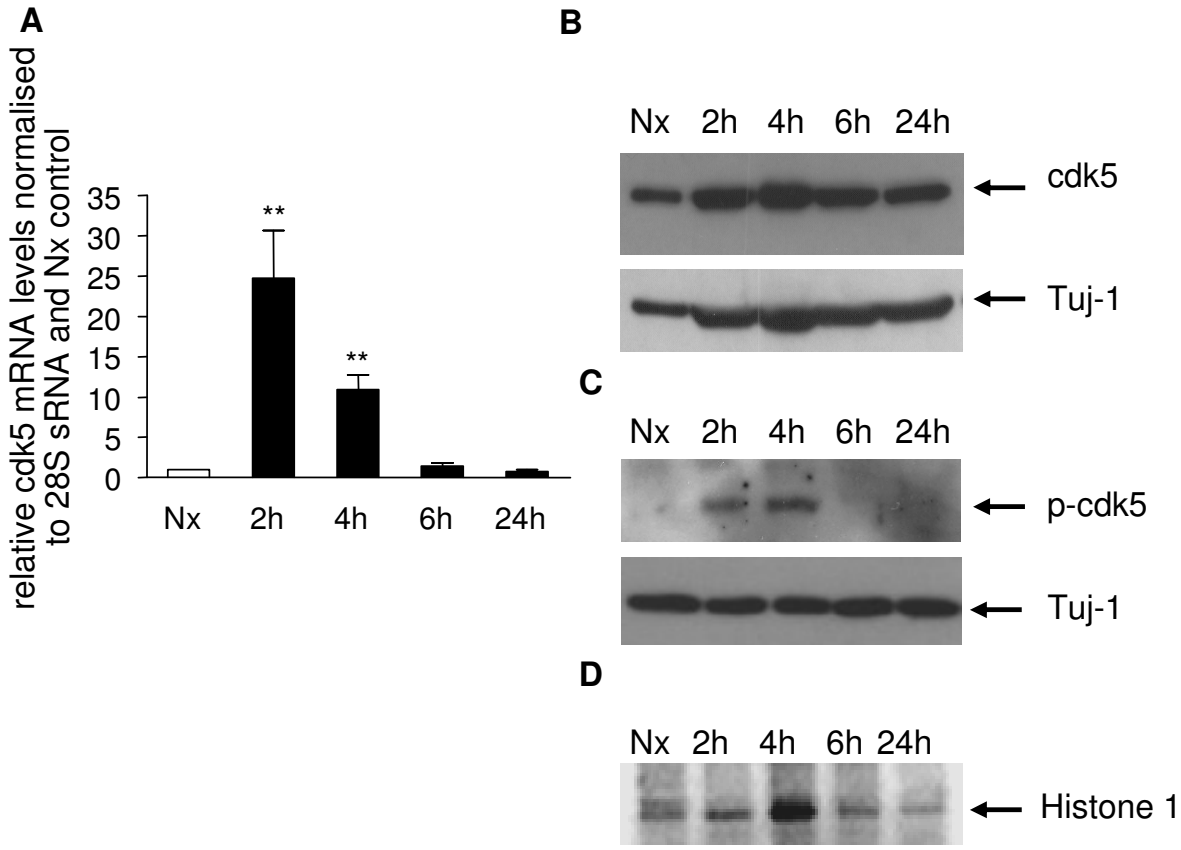
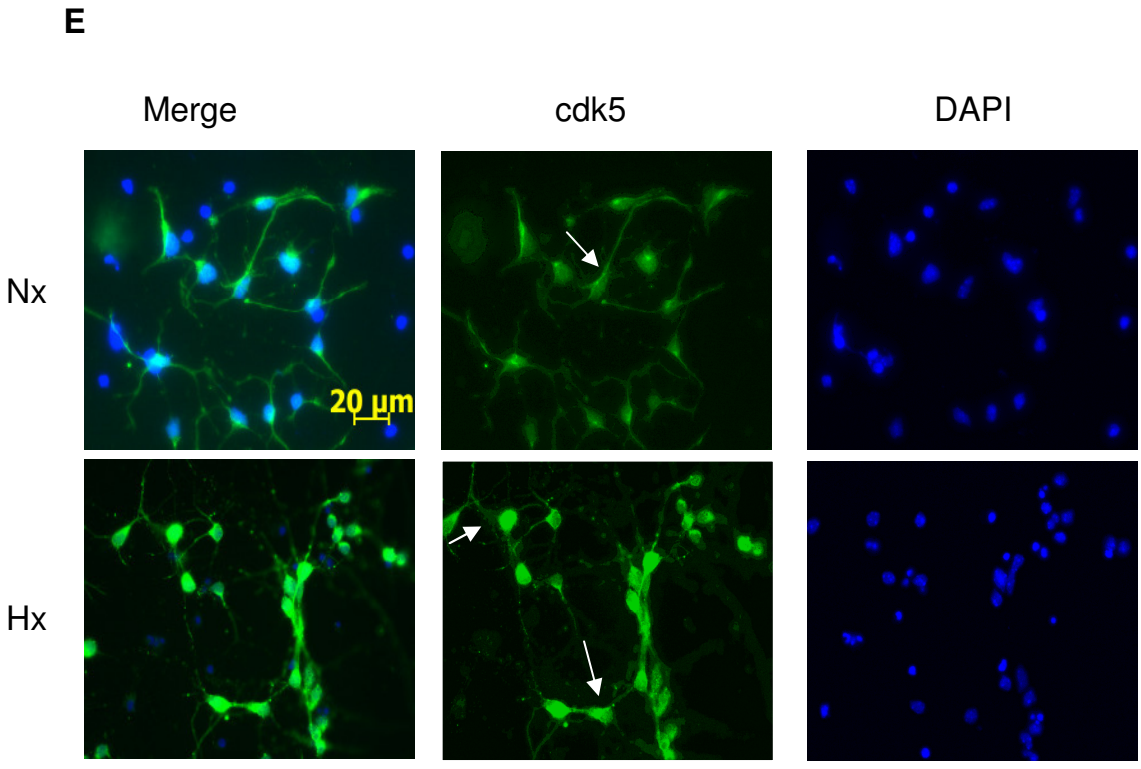
Fig. 3


A
relative cdk5 mRNA levels normalised to 28S sRNA and Nx control
35
30
25
20
15
10
5
0
**
**
Nx
2h
4h
6h
24h
B
Nx 2h 4h 6h 24h
cdk5
Tuj-1
C
Nx 2h 4h 6h 24h
p-cdk5
Tuj-1
D
Nx 2h 4h 6h 24h
Histone 1


E
Merge
cdk5
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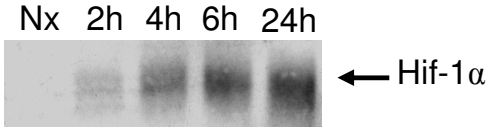
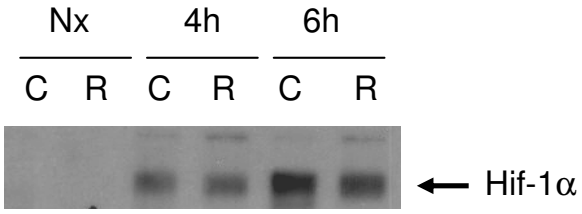
Nx
Hx
20 µm

Fig 4

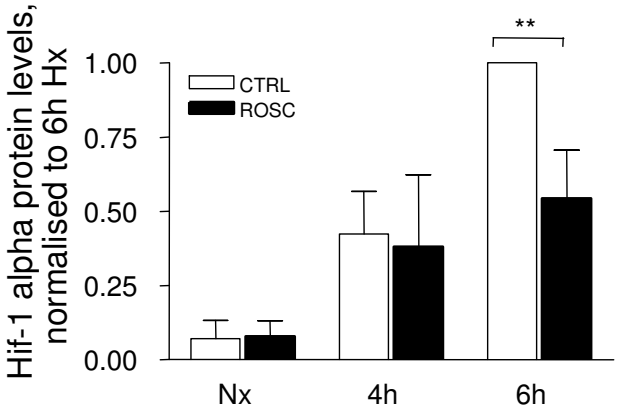
A


Nx 2h 4h 6h 24h
Hif-1α

B


Nx 4h 6h
C R C R C R
Hif-1α

C


Hif-1 alpha protein levels, normalised to 6h Hx
1.00
0.75
0.50
0.25
0.00
CTRL
ROSC
**
Nx
4h
6h

D

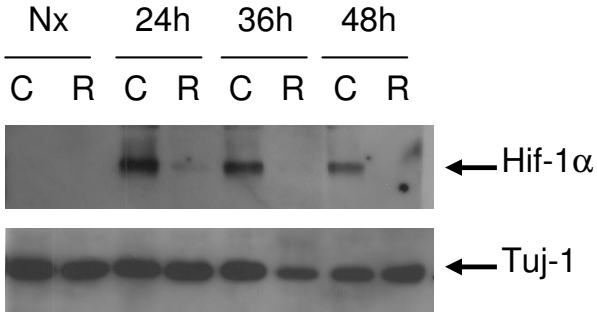

Nx 24h 36h 48h
C R C R C R C R
Hif-1α
Tuj-1

Fig 5

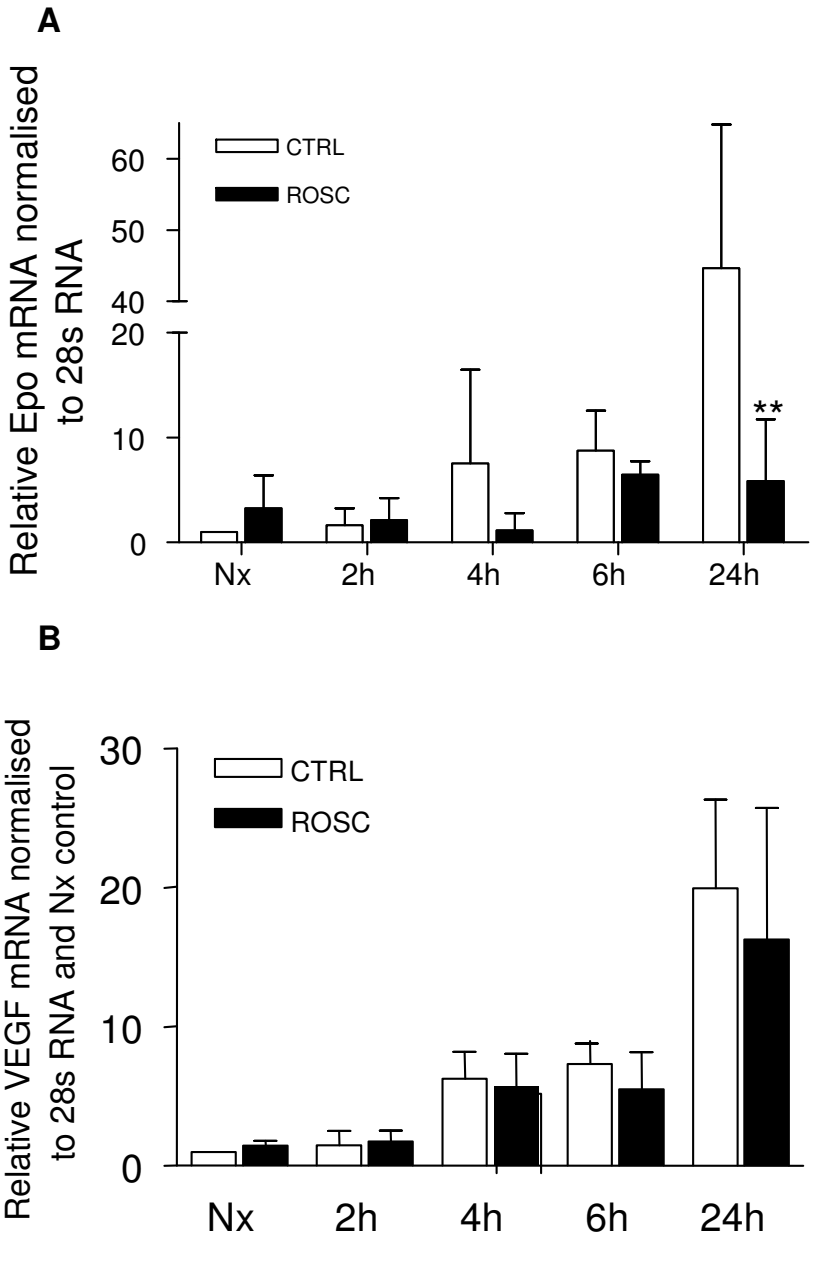
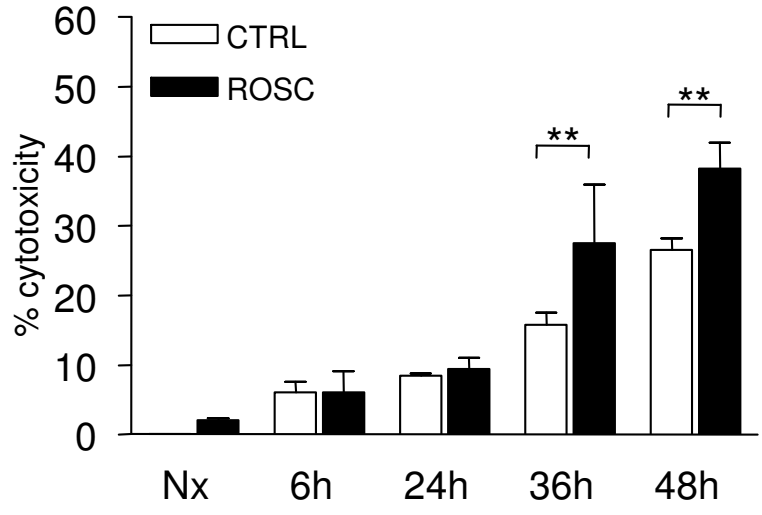

A
Relative Epo mRNA normalised to 28s RNA
CTRL
ROSC
60
50
40
20
10
0
**
Nx
2h
4h
6h
24h
B
Relative VEGF mRNA normalised to 28s RNA and Nx control
CTRL
ROSC
30
20
10
0
Nx
2h
4h
6h
24h

Fig.6


% cytotoxicity
CTRL
ROSC
60
50
40
30
20
10
0
**
**
Nx
6h
24h
36h
48h

Astrocyte responses to injury: simultaneous cell death and proliferation

Journal:	*AJP: Regulatory, Integrative and Comparative Physiology*
Manuscript ID:	draft
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Schmid-Brunclik, Nicole; University of Zurich, Institute of Veterinary Physiology Antoniou, Xanthi; University of Zurich, Institute of Veterinary Physiology Gassmann, Max; University of Zürich, Institute of Veterinary Physiology Ogunshola, Omolara; University of Zurich, Institute of Veterinary Physiology
Key Words:	oxygen deprivation, blood brain barrier, cell death, proliferation, VEGF

ASTROCYTE RESPONSES TO INJURY: SIMULTANEOUS CELL DEATH AND PROLIFERATION.

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Number of figures: 8

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ABSTRACT

Loss of blood brain barrier (BBB) integrity is a characteristic of many pathological brain diseases including stroke. Astrocytes play a central role in BBB function, their disturbance causing microvascular damage and significantly disrupting brain homeostasis. To observe how insult severity modulates their response, astrocytes were subjected to different degrees of injury. Primary cells were exposed to normoxic (21% O_2), hypoxic (1% O_2) or near anoxic (<0.1% O_2) conditions in presence or absence of glucose and the temporal induction of HIF-1α and its target gene VEGF were monitored. Hypoxic exposure for over 72 hours did not detectably induce HIF-1α, however near anoxic conditions stabilised the protein and provoked DNA binding activity. Glucose deprivation alone also stabilised HIF-1α and subsequent O_2 withdrawal resulted in faster accumulation. Notably, VEGF levels were increased by hypoxic exposure, in the absence of HIF-1α expression, and glucose withdrawal further accelerated VEGF secretion. Cell death was significantly initiated after 6h of combined oxygen glucose deprivation, and surprisingly, astrocyte proliferation increased concomitantly. Thus high HIF-1α levels correlated with both cell death and proliferation. These data suggest that protection of astrocytes during chronic injury (as occurs in clinical hypoxic/ischemic insults) may protect BBB integrity.

Key words: oxygen deprivation, blood brain barrier, cell death, proliferation, VEGF

Running Title: Anoxia, HIF-1 and astrocytes

INTRODUCTION

Cerebral ischemia disrupts the BBB thereby irreversibly damaging neurons and astrocytes (20, 23) and often causing lethal cerebrovascular diseases. The blood brain barrier (BBB) consists of highly specialised vessels with a specific architecture that maintains homeostasis of the brain environment. Astrocytes, the most abundant glial cells in the brain, contact the brain vasculature with their endfeet processes and this interaction is thought to induce endothelial tight junction formation and decrease permeability of the BBB (38). Astrocytes are known to be more resistant to oxidative stress than neurons and play a neuroprotective role through their ability to take up potassium and glutamate and release mitogenic factors (33). However in response to injury, astrocytes retract their end feet from vessels resulting in increased permeability, as well as proliferate giving rise to a glial scar (17, 25, 35). Since both of these events can have significant detrimental effects on the brain understanding how these complex cells respond to insult is important to prevent vascular leakage and brain damage.

Hypoxic/ischemic insults cause activation of adaptive mechanisms and alteration of gene expression within the injured areas to combat the progression of pathological events (1). At the cellular level an oxygen sensing system is crucial to enable rapid adaptation to altered oxygen tensions. A well-characterised molecular pathway that mediates oxygen sensing occurs through the induction of the transcription factor HIF-1 (hypoxia-inducible factor-1). HIF-1 consists of an oxygen-dependent α subunit and a constitutively expressed β subunit. Both subunits are basic-helix-loop-helix (bHLH) proteins of the PAS family and complex binding enhances the transcription of target genes involved in glycogenesis, erythropoiesis and angiogenesis (12). HIF-1α is mainly regulated at the protein level by O_2. During normal O_2 supply HIF-1α is hydroxylated by oxygen dependent prolyl-4-hydroxylases, ubiquitinated and rapidly degraded by the proteosomal system (8). Hypoxia stabilises HIF-1α and promotes its translocation to the nucleus, accumulation and dimerisation with HIF-1β ultimately

enhancing transcriptional activity of its target genes through binding to its HRE (hypoxia response element).

A major HIF-1 target gene known to have a cytoprotective role is the potent angiogenic molecule VEGF (vascular endothelial growth factor) (10). VEGF binds with high affinity to two receptor tyrosine kinases, flt-1 (VEGFR-1) and flk-1 (VEGFR-2), and activates downstream pathways (reviewed in (28)) promoting angiogenesis during development, as well as (patho)physiological events such as pregnancy, wound healing, rheumatoid arthritis, cardiovascular disease and cancer (9, 26). Notably, astrocytes secrete VEGF under normal physiological conditions but hypoxia further induces VEGF mRNA and protein levels (5, 16, 30) causing increased vascular permeability and proliferation of glial cells (17, 39).

Since astrocytes play a crucial role in brain homeostasis, their response to hypoxic/ischemic insult deserves further investigation. The aim of the present study was to investigate and compare the astrocytic response to mild and severe hypoxic/ischemic insults. Specifically we examined the influence of oxygen- and glucose-deprivation on HIF-1 stabilisation and expression of VEGF in primary rat astrocytes. We also monitored proliferation, induction of cell death pathways and alteration of ATP levels during injury. We show that astrocytes do not induce HIF-1α under standardly used hypoxic conditions (1% O_2) but only when exposed to near anoxic conditions. ATP levels and cell viability are well maintained in these cultures under these conditions but deprivation of both glucose and oxygen induce cell death within 6 hours. Interestingly at this time the highest proliferative rates of these cells was also observed.

MATERIALS AND METHODS

Primary culture of astrocytes

Primary astrocytes were prepared from newborn Wistar rat pups as previously described (5). Briefly neonatal rats were anesthetised by hypothermia, decapitated and cerebral cortices removed. Meninges were removed and cortices minced and placed in ice cold buffer (Krebs solution 120mM NaCl, 3mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 25mM $NaHCO_3$, 11mM glucose and 0.6g/l BSA). Homogenised tissue was digested with 7.5mg trypsin for 15 min at 37°C and dissociated cells were cultured in DMEM containing 10% FBS, 150mg/l L-Glutamine and 50mg/ml Gentamicin at 37°C. The culture medium was changed every 4-5 days. After 7- 10 days cultures displayed uniform (> 96%) glial fibrillary acidic protein (GFAP) immunoreactivity. Astrocytes were passaged a maximum of 3 times and used at 80-90% confluency.

Hypoxic and glucose deprivation experiments

For all experiments, primary astrocytes were incubated under normoxic (21% O_2), hypoxic (1% O_2) or near anoxic (<0.1% O_2) conditions in a purpose built humidified glove-box incubator (In vivo 400, Ruskinn technologies, UK). Glucose deprivation experiments were carried out with glucose-free DMEM (Life technologies).

Western blot analysis

Cells were scraped into lysis buffer (0.27 M Sucrose, 2mM EDTA (pH 8.0), 0.1% NP-40, in 0.6M KCl, 150mM NaCl, 150mM HEPES (pH 7.5)) and centrifuged for 10 min at 16'000 rcf. Cytoplasmic fractions were frozen and nuclear pellets resuspended in nuclear extraction buffer (20mM HEPES (pH 7.5), 400mM NaCl,1mM EDTA(pH8.0)) and left 15 min on ice. Nuclear extracts were obtained after centrifugation (16'000 rcf) for 10 min and separated by denaturing SDS-PAGE. After transfer to nitrocellulose membranes were incubated in 4%

milk in PBS for 1h at room temperature then were incubated overnight at 4°C with antibodies against HIF-1α IgY (4) (diluted 1:100 in PBS). Following washes, membranes were incubated with a secondary HRP-conjugated antibody for 1h at room temperature. Membranes were exposed to X-ray film after luminescent detection (25µl of 90mM coumaric acid in DMSO, 50µl of 250mM luminol in DMSO in 10ml of 100mM Tris, pH 8.5). For loading controls blots were stripped and re-probed for the nuclear protein Sp-1. Results were quantified by densitometry using BioRad Quantity One software (BioRad laboratories AG, CH).

VEGF ELISA

Quantitation of VEGF was performed using a VEGF ELISA kit (R&D Systems GmbH, Germany) according to manufacturers instructions. Optical density was measured using a microplate reader (Multiskan RC; Thermo Labsystems, Finnland) at 450nm with wavelength correction at 570nm.

EMSA (electrophoretic mobility shift assay)

EMSA was performed with nuclear extracts as previously described (15). Oligonucleotide probes derived from the EPO enhancer gene 5'-GCC CTA CGT GCT GTC TCA-3' (3'EPO enh) and 5'-TGA GAC AGC ACG TAG GGC-3' (5'EPO enh.rev) were synthesized by Microsynth (Balgach, Switzerland). End-labelling was performed with T4 PNK (Fermentas, LabForce AG, Nunningen, Switzerland) at 37°C for 1h. Anti-sense oligonucleotides were added in 2-fold molar excess and annealed at 95°C for 3min. Samples were loaded on a 4% non-denaturing gel and electrophoresis was performed at 200V in 1xTBE buffer at 4°C. After drying the gel was visualised using BioRad Molecular Imager FX.

In situ labelling of fragmented DNA

TUNEL labelling was carried out according to the manufacturers instructions (Roche Biochemicals, Penzberg, Germany). Astrocytes grown on cover slips and subjected to different conditions were fixed for 30 min in 4% paraformaldehyde. Staining was carried out in a humidified atmosphere for 1 hour and nuclei were counterstained with DAPI for 3min. Samples were analysed by fluorescence microscopy.

Proliferation Assays

AlamarBlue assay was used to quantitatively measure astrocyte proliferation. Experiments were performed according to the manufacturers instructions (AlamarBlue; Biosource, Camarillo, CA). In brief primary astrocytes were seeded on 96-well plates at a density of 2000 cells per well and incubated overnight. Four hours prior to measurement 20µl of AlamarBlue dye was added to each well and plates were incubated under normoxic, hypoxic or anoxic conditions with or without glucose as described above. Absorbance was measured with a plate reader (Multiskan RC; Thermo Labsystems, Finland) at 540nm subtracting the background absorbance at 630nm.

Thymidine incorporation assay was used as an alternative method to AlamarBlue. Cells were grown to 90% confluency on 24 well plates overnight. At time 0, individual plates were exposed to normoxia, hypoxia or near anoxic conditions in presence or absence of glucose after addition of 1Ci [3^{H}]thymidine. After 6 hours the astrocytes were washed with ice cold PBS, typsinised and harvested onto glass fiber filters (Wallac, Hünenberg, Switzerland). The amount of incorporated radioactivity was counted using a Wallac counter.

In all proliferation assays individual experiments were performed 5 times in quadruplicate and data was converted to fold change compared to normoxic cells grown in glucose containing media.

ATP measurements

ATP was measured with an ATP bioluminescent assay kit (Sigma chemical company, Buchs, Switzerland). Cells were lysed in buffer (105mM NaCl, 1% Triton X-100, 1% NP-40, 50mM Tris) for 10 min before centrifugation (10 min at 16'000 rcf). The supernatant was mixed with TCA 5% (1:1 v/v) to block residual ATPase activity, centrifuged for 1 min at 16'000 rcf and neutralized with saturated Tris solution to pH 7.8. Sample was added to diluted ATP assay mix (1:1 ratio) and luminescence measured using Berthold luminometer (Detection systems GmbH, Pforzheim, Germany). Cellular ATP levels were determined from a standard curve and normalised to total protein concentrations.

Statistical analyses

Graphics and statistical analyses were performed using Microsoft excel software. All results are expressed as fold change over normoxic values. Statistical significance ($P< 0.05\%$) was calculated using student T-test.

RESULTS

HIF-1α protein stabilisation in astrocytes occurs only after severe oxygen deprivation

Many reports demonstrate that HIF-1α protein stabilisation occurs immediately after hypoxic exposure (1% O_2) in various cell lines (14, 36). To observe time-dependent HIF-1α accumulation in primary cultured astrocytes, nuclear protein extracts were prepared from normoxic cells (21% O_2) and cells exposed to hypoxic (1% O_2) or near anoxic (<0.1% O_2) conditions. Surprisingly HIF-1α protein could not be detected by Western blot analysis under hypoxic conditions (fig.1a) even after 72 hours of exposure. Only near anoxic incubation induced the accumulation of HIF-1α protein in a time-dependent manner (fig.1b). Subsequent reoxygenation (21% O_2) for 5 hours (fig.1, 5R) abrogated HIF-1α protein stabilization. As

expected HIF-1β levels remained constant at all time points investigated (data not shown). To investigate whether the induced HIF-1α was active and capable of binding target HRE sequences we performed EMSA analyses. Under normoxic and hypoxic conditions no HIF-DNA binding complex was detected, however a weak binding signal was identified after 2h anoxia (fig.1c). The intensity of this band increased with extended incubations corresponding to Western blot data and confirming that astrocytes stabilise HIF-1α only after severe oxygen deprivation.

VEGF protein is induced in the absence of HIF-1α expression

VEGF, a major HIF-1α target gene, is strongly induced by hypoxia (3, 30). To determine if VEGF is upregulated in astrocytes in accordance with HIF-1α stabilisation, VEGF protein levels in conditioned media and cell lysates were measured by ELISA. Figure 2a shows increased VEGF expression in cytoplasmic fractions following oxygen deprivation. Notably, hypoxic incubation induced VEGF protein levels immediately reaching maximum after 6h (4.5 fold increase over normoxic levels, **p=0.001) even though HIF-1α was not detected under these conditions. Interestingly, cytoplasmic VEGF induction after near anoxia was similar until 6h but by 24h a more than 5 fold increase was observed (fig.2a) indicating that only prolonged anoxia (>24h) significantly induces VEGF over hypoxic conditions. Time-dependent secretion of VEGF into the culture media was also monitored (fig.2b) but no alterations between hypoxia and anoxia were observed until 24h. Thus astrocytes produce similar amounts of VEGF irrespective of the insult severity during acute insult, but chronic exposure significantly elevates VEGF production and secretion.

Immediate HIF-1α protein stabilization following an ischemic insult in vitro

An ischemic event such as stroke deprives cells of both oxygen and glucose. To mimic *in vivo* models of ischemia, astrocytes were placed in glucose-free media immediately prior to anoxic exposure. Glucose deprivation alone caused immediate stabilization of basal HIF-1α protein levels (fig.3a, 0h) and additional hypoxia further induced nuclear HIF-1α protein accumulation in a time-dependent manner. Near anoxic glucose-deprived astrocytes stabilised HIF-1α even more rapidly with a significant increase observed after just 2 hours (fig. 3a) and 3 fold induction compared to hypoxic samples after 6h (fig. 3b). Note that 24 hours of anoxic incubation protein could not be extracted implying major cell death at that time.

Although glucose withdrawal alone resulted in immediate basal HIF-1α stabilisation, prolonged glucose deprivation did not further increase HIF-1α accumulation. Western blot analysis of astrocytes incubated in glucose-free medium under normoxic conditions for extended periods (6, 24 and 48 hours) showed no additional increase in HIF-1α accumulation (data not shown). Glucose replenishment for 3 hours also had no effect. Thus although glucose withdrawal stabilises low basal HIF-1α levels in astrocytes additional oxygen deprivation is required to further induce protein accumulation.

Severe insult stimulates rapid and increased secretion of VEGF from astrocytes

Although glucose and oxygen deprivation increased cytoplasmic VEGF levels 8-10 fold over normoxic controls after 6 hours (fig.4a), no difference was observed between hypoxic and anoxic samples. Furthermore VEGF protein levels were not significantly enhanced compared to astrocytes cultured in glucose-containing media (fig.2a) until the 24h timepoint. Accumulation of secreted VEGF into the media however did increase in a time-dependent manner with 3 fold higher levels reached in anoxic compared to hypoxic glucose deprived cells after 6 hours (fig.4b). Therefore despite VEGF cytoplasmic levels of VEGF being

maintained for up to 6h, secretion of VEGF is significantly enhanced in oxygen and glucose-deprived astrocytes.

Increased severity of insult stimulates astrocytic proliferation

Since injury induces increased proliferation of glial cells and formation of a glial scar *in vivo* (17, 39), we investigated whether the severity of insult can directly modulate the proliferative rates of astrocytes. AlamarBlue and thymidine incorporation assays (fig.5a&b respectively) were used to determine the proliferative capacity of astrocytes in the presence and absence of glucose for 6 hours. Of note, different severities presented distinct proliferation patterns (fig.5). No significant difference was observed between normoxic and hypoxic glucose-containing cells but anoxia stimulated increased proliferation significantly (~30%). Surprisingly glucose withdrawal further induced proliferation. Normoxic glucose deprivation caused a 40% increase in proliferation over normoxic glucose-containing cells whereas near anoxia combined with glucose deprivation increased proliferation a further 2 fold using both techniques. In conclusion, increased severity of insult augmented proliferation of astrocytes *in vitro*.

Only combined oxygen and glucose withdrawal induces cell death in astrocytes

Increased proliferation during severe insult suggested that astrocyte survival is preserved and cell death is suppressed during this time. In the presence of glucose, astrocytes were TUNEL negative at all time points without any visible nuclear condensation (data not shown) indicating the cells survive oxygen deprivation well. Somewhat surprisingly, cell death processes in hypoxic glucose-deprived astrocytes were identified only after 24 hours exposure (approximately 10% TUNEL positive nuclei in fig.6 top panels). Anoxia combined with glucose deprivation caused earlier (6h) TUNEL positive staining and nuclear condensation (fig.6 middle panels). By 24h virtually all astrocyte nuclei (90%) were TUNEL positive (fig.6

bottom panels) with clear nuclear condensation and cell shape disruption. Thus prolonged severe insult initiated death pathways in virtually all astrocytes.

ATP levels are preserved despite oxygen and glucose withdrawal.

To get a handle on how astrocytes can survive oxygen and/or glucose deprivation for long periods of time without undergoing cell death we measured cellular ATP levels (fig.7). In glucose containing media (fig.7a) normoxic ATP levels were virtually constant for the duration of the experiment. Hypoxia and anoxia reduced ATP levels by 50-70% within the first 6-24 hours (fig.7a) but were subsequently maintained for the duration of the experiment. Glucose deprivation (fig.7b) elicited a different response. A 50% decrease in normoxic ATP levels occurred within 6h glucose withdrawal before recovering to 70% at 48h and hypoxic ATP levels were maintained slightly better than normoxic cells at all time points, indicating that hypoxia has no additional detrimental effect on ATP levels in the absence of glucose. Anoxia combined with glucose deprivation resulted in a dramatic 70% decrease in ATP levels already at 6h that were virtually zero at 24h. Therfore although astrocytes are able to tolerate oxygen and glucose deprivation, simultaneous long-term withdrawal is ultimately lethal after 6 hours.

DISCUSSION

The ability of cells to cope with reduced oxygen levels is critical to their survival but hypoxic tolerance varies greatly amongst different cell types. It is well established that during hypoxia or ischemic insult loss of astrocytes causes microvascular damage and exacerbates disturbance of brain homeostasis (38). In this work we have investigated the astrocytic response to different degrees of injury with emphasis on the temporal induction of HIF-1α and its target gene VEGF. We also examined the extent of cell death and ATP maintenance

during insult. We show that astrocytes, in contrast to most other cell types, require severe oxygen deprivation to prevent HIF-1α degradation and are able to survive severe ischemic conditions for up to 6 hours prior before undergoing cell death.

It has been documented that astrocytes are less susceptible to injury than other brain cells such as neurons (18). Our work confirms observation this since stabilisation of the HIF-1 heterodimeric complex (and thus a full stress response) in astrocytes requires lower O_2 levels compared to most studied cells. The requirement of severe oxygen deprivation to stabilise HIF-1α may be explained by the essential role of astrocytes in BBB maintenance. It is fitting that astrocytes are not sensitive to small changes in oxygen levels as this could have disastrous consequences on BBB function and neuronal homeostasis. However, HIF-1 not being induced by hypoxia does not mean astrocytes are insensitive to changes in O_2 tension – indeed it has been shown that although mild hypoxia does not affect astrocyte survival, substantial differences in metabolism still occur that contribute to their adaptation (34). HIF-1α is involved in the regulation of glucose metabolism (27) and as such increased HIF-1 stabilisation that we observe after severe oxygen deprivation is a likely prerequisite for astrocytes to increase their glycolytic capacity during insult (18). In agreement with this, we observed that glucose withdrawal alone also induced low basal HIF-1α stabilisation and astrocytes were far more sensitive to oxidative stress in combination with glucose withdrawal. It has been proposed that abundance and hydroxylase capacity of prolyl hydroxylases may fine-tune the dynamic range of oxygen sensitivity and be rate-limiting for HIF-1 degradation (32) thus defining the cellular response to insult. However existence of such a cellular mechanism is still to be clarified.

The HIF-1α target gene VEGF is induced by hypoxic/ischemic insult in astrocytes (2) but is also constitutively expressed at lower levels under normoxic conditions (3, 21). Indeed hypoxic exposure moderately increased cytoplasmic accumulation and basal release of VEGF in our astrocyte cultures in a time-dependent manner. *In vivo* this response is probably

advantageous since enhanced VEGF release, in addition to protecting neighbouring cells through paracrine mechanisms (5, 37), may protect astrocytes themselves from cell death through autocrine signalling. Notably this elevation of VEGF occurred in the absence of detectable HIF-1α stabilisation suggesting a HIF-1-independent regulatory mechanism during mild oxygen deprivation. Although an obvious candidate is HIF-2α, during our study constitutive HIF-2α expression levels were unaffected by hypoxia (data not shown) in agreement with others (22). Thus how VEGF is hypoxically induced in the absence of HIF-1 remains intriguing and deserves further investigation.

Glucose-free astrocyte conditioned media contained dramatically elevated VEGF concentrations compared to glucose-containing conditioned media in agreement with other studies of hypoglycaemic VEGF induction in glioblastoma and monocytes (29, 31). Now we show that increased severity of insult progressively augments VEGF release without affecting cytoplasmic stores. Although moderate upregulation of VEGF could enhance survival of surrounding cells, a rapid massive increase in VEGF concentrations may have an overall negative effect on the BBB causing increased permeability and vascular remodelling. Indeed the temporal upregulation of VEGF in our model reflects the profile of BBB leakage *in vivo* with VEGF induced as early as 1–3 h after focal cerebral ischemia onset and peaking at 24–48h (6, 24). Taken together this data implies that astrocytes do indeed have the capacity to secrete significant amounts of VEGF during ischemic injury that can induce BBB leakage.

Studies by Jelluma et al (13) showed that astrocytes proliferate during glucose withdrawal and our data further indicates that injury severity is a strong proliferative stimulus. As VEGF promotes astrocyte proliferation (17, 19), enhanced secretion that we observe is likely to be responsible for the increased proliferation during glucose withdrawal, although other factors are not ruled out. Both hypoxia and ischemia also activate and promote proliferation of resident astrocytes *in vivo* (25, 40), a process often referred to as astrogliosis or glial scarring. Suggested to be a reparative process to restore homeostasis through isolation of the damaged

region, a glial scar may also detrimentally interfere with subsequent neural repair or axonal regeneration, cause release of microglial-associated inflammatory factors as well as alter cellular interactions at the BBB. Thus preventing proliferation during the first 6 hours of injury may be of significant benefit as also indicated by Di Giovanni et al who showed that flavopiridol administration 30 minutes after traumatic brain injury significantly reduced lesion volumes in rats (7).

Paradoxically, we show that increased severity of insult stimulated astrocyte proliferation despite reduced ATP content. Naturally, constant ATP levels are required to ensure cell viability during stress (11) and in most experimental paradigms astrocytes crucially maintained their ATP levels at or above 50%. Remarkably cell survival was compromised only when oxygen and glucose deprivation was performed in parallel. Anoxic glucose-deprived cells were unable to recover from a 70% drop in ATP concentrations, despite high HIF-1α expression, culminating in cell death. Thus 6h of extreme insult significantly lowered astrocyte viability as was confirmed by the detection of TUNEL-positive cells at this time. Therefore a strong correlation exists between proliferation and cell death during severe insult. That proliferation is high in cells ultimately pre-destined to die may either be a last effort to restore homeostasis or a suicide mechanism. How hypoxia/ischemia modulates activation and/or proliferation of astrocytes and whether HIF-1 itself plays a direct role is currently under investigation.

In summary, cultured astrocytes do not stabilise HIF-1α protein under standard hypoxic conditions (1% O_2) but require severe oxygen deprivation for induction to occur. Surprisingly, VEGF regulation in mild conditions may not be controlled by HIF-1. Astrocyte HIF-1α stabilisation directly correlated with simultaneous induction of cell death and proliferation pathways. To conclude, this study highlights how astrocytes respond to different degrees of insult. Since impairment or alteration of astrocyte function can cause microvascular damage

and accelerate neuronal death/hyperactivation, such knowledge may be useful for developing selective treatment for brain recovery after injury.

ACKNOWLEDGEMENTS

The authors thank Dr. Martijn Moransard for critical reading of the manuscript. Supported by Swiss National Science Foundation, Stiftung 3R and EUROXY (EU) grants to MG and OOO.

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FIGURE LEGENDS

Figure 1: HIF-1α is stabilized only during severe oxygen deprivation. Representative Western blot of HIF-1α protein accumulation in astrocytes following hypoxic (1% O_2) (a) and near anoxic (<0.1% O_2) incubation (b). Sp1 was used as a loading control (b). Representative HIF-1α EMSA on nuclear protein extracts isolated from primary astrocytes exposed to normoxic and near anoxic conditions (c). HIF-DNA binding activity was observed only after anoxic incubation of astrocytes and five hours of reoxygenation (5R) abrogated HIF binding.

Figure 2: VEGF levels in astrocyte cytoplasmic fractions and media by ELISA. Cytoplasmic VEGF levels increased in all cells subjected to oxygen deprivation (a). Near anoxic conditions (<0.1% O_2) induced the most significant increase over control levels but only after 24 hours. Significantly lower amounts of VEGF are released into the media (b). (*=p<0.05, **=p<0.005; n=5).

Figure 3: Combined oxygen and glucose withdrawal strongly induces HIF-1α in astrocytes. Representative Western blot demonstrates time-dependent nuclear HIF-1α accumulation following both hypoxic and anoxic incubation after glucose withdrawal (a). Sp1 reblot was used as a loading control. Interestingly, sole glucose withdrawal also induced stabilization of HIF-1α (a, 0h lanes). Quantitation of Western blots (b) shows anoxic glucose deprivation significantly increased HIF-1α levels over hypoxia at all time points. Anoxic exposure was not possible for longer than 6h due to cell death. (**=p<0.005; n=3).

Figure 4: Combined glucose and oxygen deprivation significantly increases VEGF secretion. Cytoplasmic VEGF protein levels in hypoxic and anoxic astrocytes deprived of glucose were similar up to 6 hours (a). By 24h, hypoxic VEGF levels increased significantly. Anoxic incubation was not possible after 6h. Anoxic glucose-deprived cells secreted more VEGF into

the culture media than hypoxic cells (b). By 24h significant VEGF protein had also accumulated in hypoxic astrocyte media. (*=p<0.05, **=p<0.005; n=3).

Figure 5: Increased severity of insult enhances astrocytes proliferation. Proliferation was determined by AlamarBlue (a) and thymidine incorporation assays (b) after exposure to normoxic, hypoxic or anoxic conditions in the presence or absence of glucose for 6h. In glucose containing cells, anoxia caused a 30% increase in proliferation compared to normoxia and hypoxia. Glucose deprivation significantly augmented proliferation of normoxic and oxygen-deprived astrocytes. Anoxic cells displayed the most robust increase of up to 2.0 fold over normoxic and hypoxic cells after 6 hours. (*=p<0.05; **p=<0.005; n=5).

Figure 6: Cell death occurs only during prolonged oxygen glucose deprivation. Isolated TUNEL-positive nuclei were detected only after 24h of hypoxic incubation (top panels). Near anoxic exposure for 6h resulted in visible nuclear condensation and positive staining of isolated cells as indicated by arrowheads (middle panels). After 24h of anoxia (lower panels) most cells were TUNEL-positive. Co-localisation of TUNEL positive nuclei (green) with DAPI nuclear stain (blue) is seen in right hand panels.

Figure 7: Astrocyte ATP levels are severely depleted after oxygen and glucose withdrawal. Intracellular ATP measurements of cells cultured in glucose containing media (a) showed decreased levels of 20 and 50% under hypoxic and anoxic conditions respectively compared to normoxic cells after 6 hours. ATP levels were then maintained between 50 and 80% for up to 48h. Additional glucose deprivation (b) resulted in a 70% decrease of ATP levels in anoxic cells. ATP levels of anoxic cells did not recover and were virtually zero after 24h. (*=p<0.05; **=p<0.005; n=4-5).

Figure 1

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Hif-1α is stabilised only during severe oxygen deprivation (i.e. near anoxic conditions)

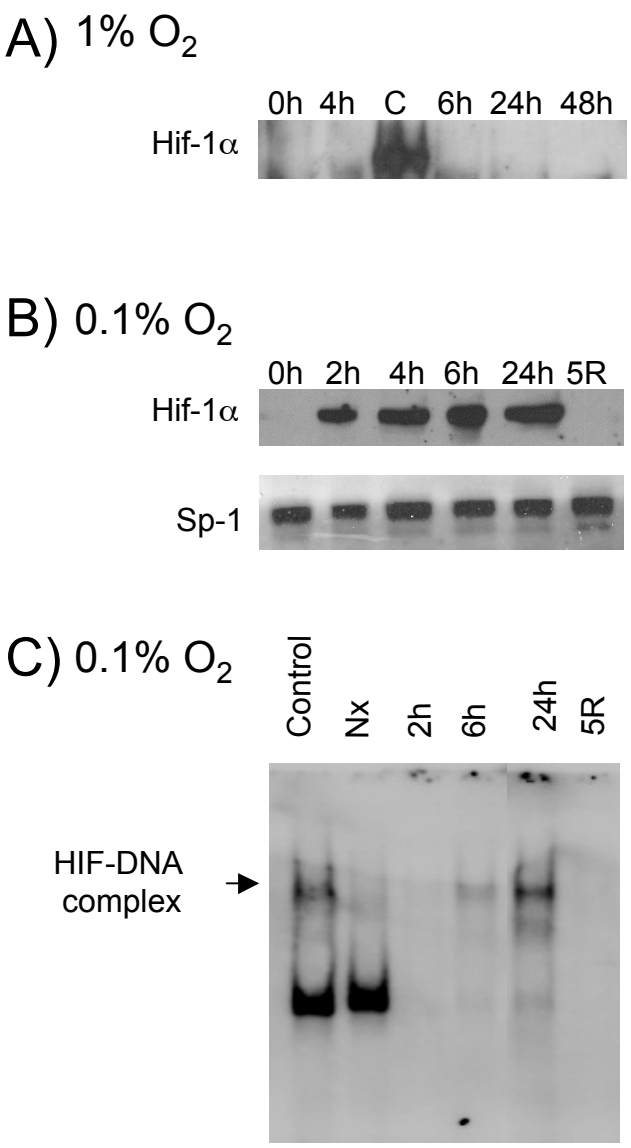

Figure 2

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VEGF levels during oxygen deprivation

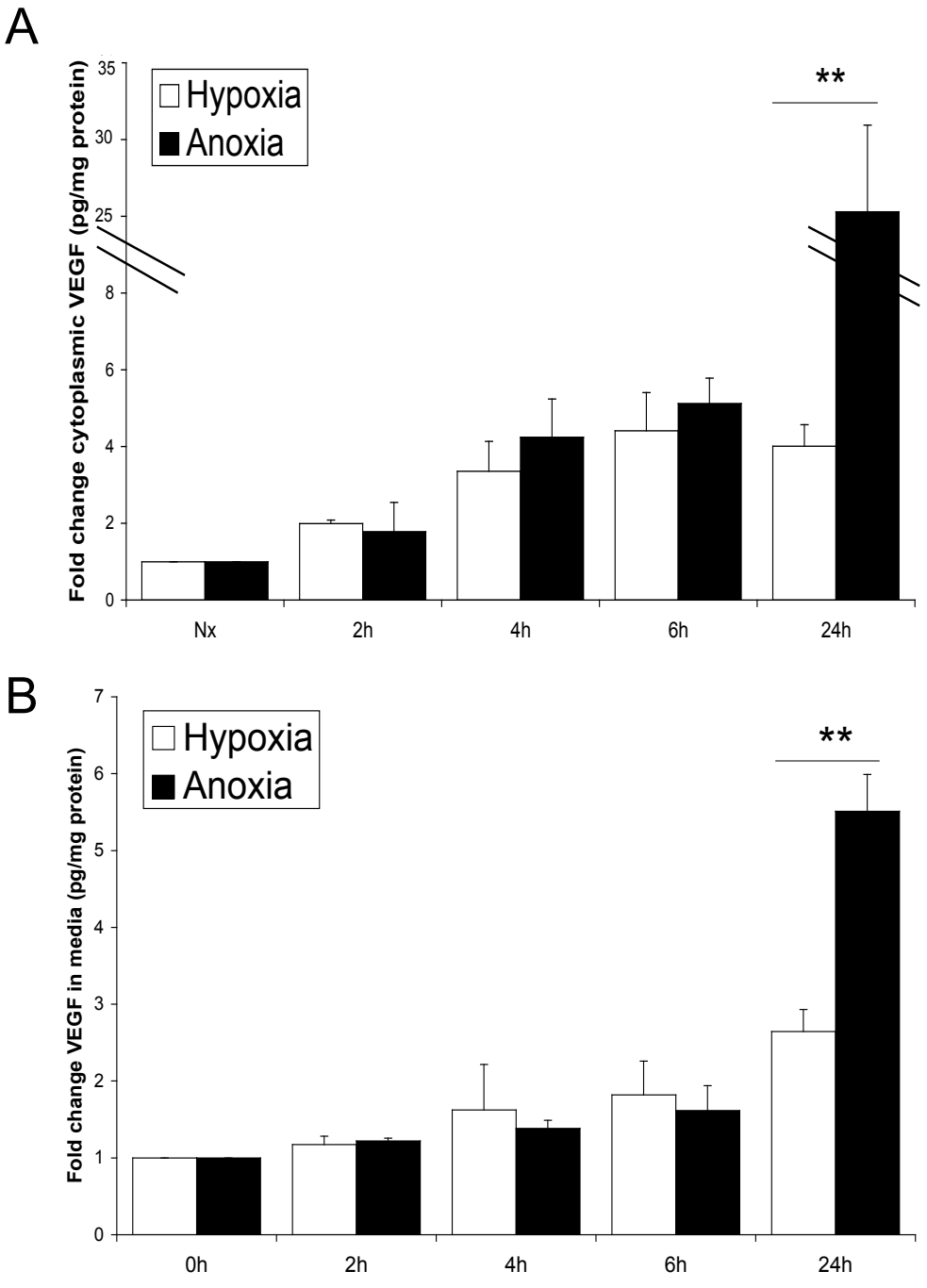

Figure 3

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Glucose deprivation further induces HIF-1α expression

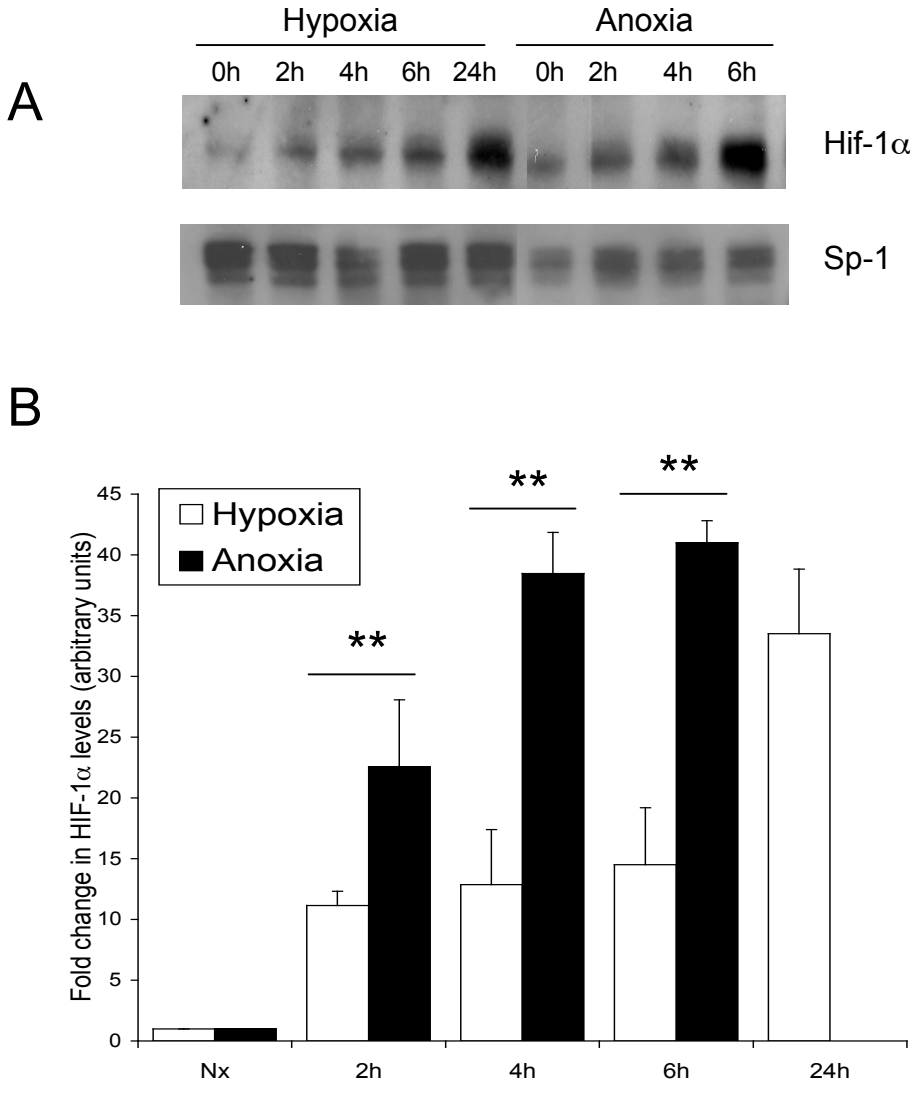

Figure 4

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Increased VEGF release during glucose deprivation

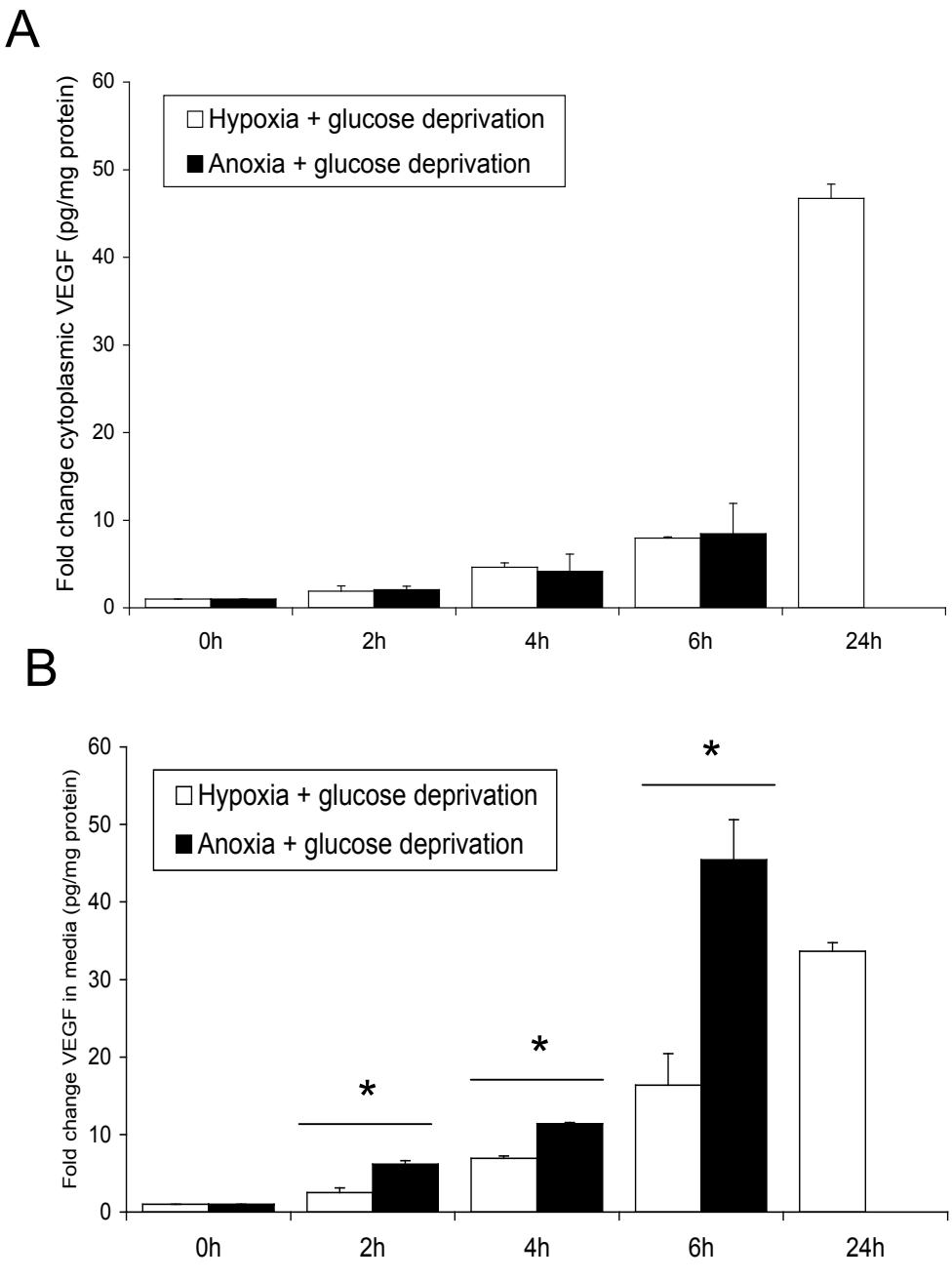

Figure 5

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Oxygen and glucose deprivation stimulate increased proliferation

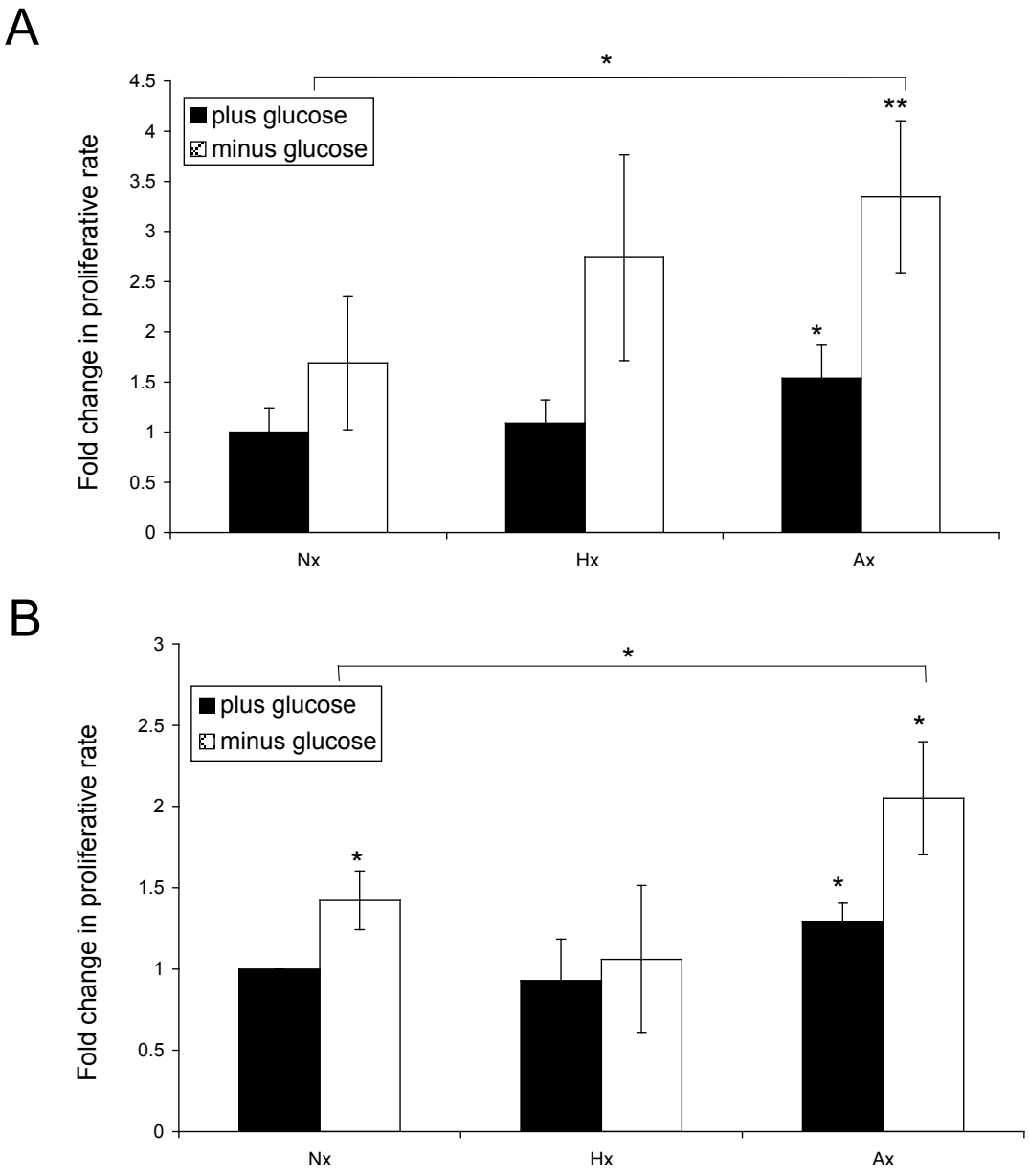

Figure 6

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Apoptosis occurs only during oxygen and glucose deprivation

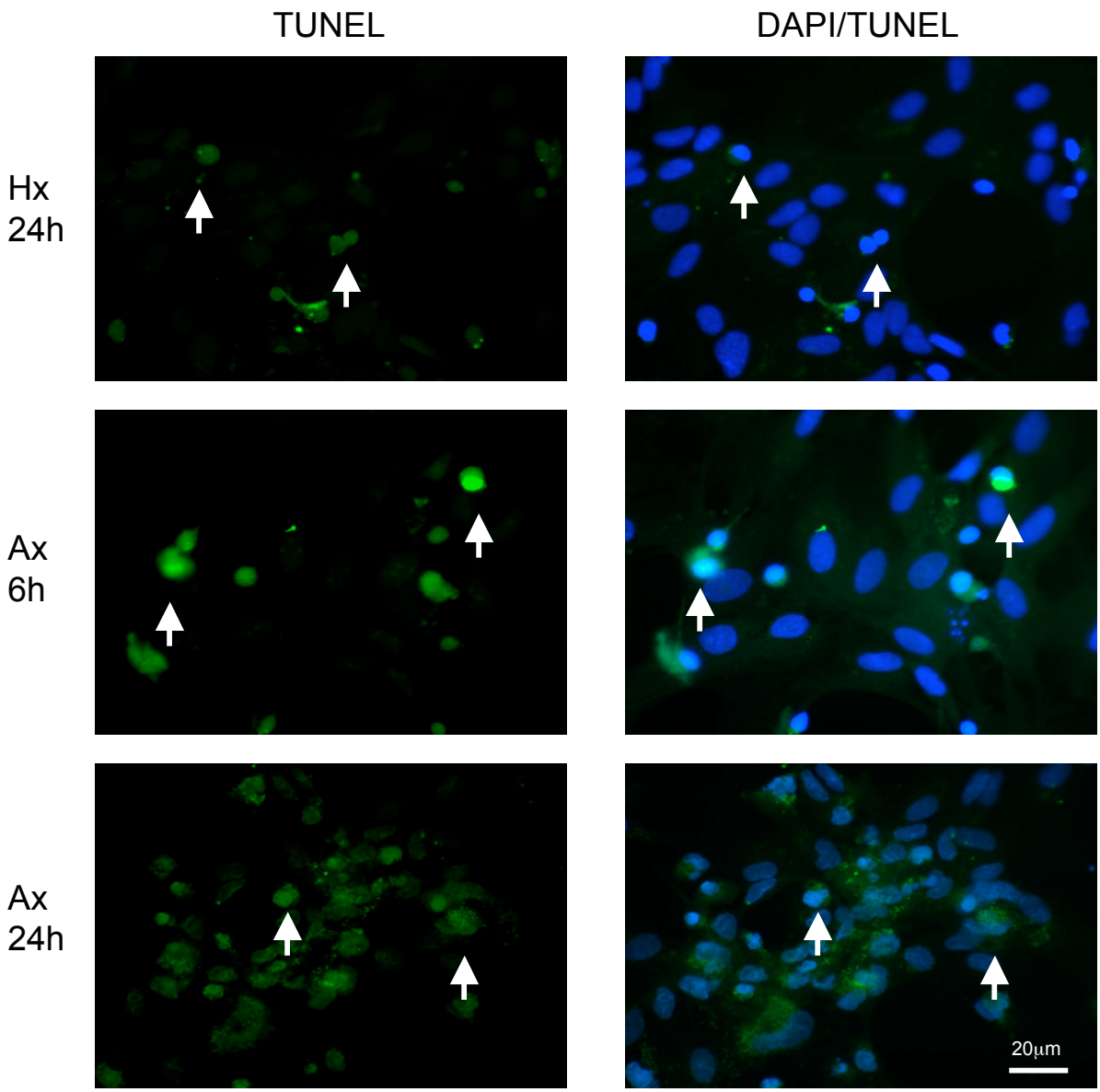

Figure 7

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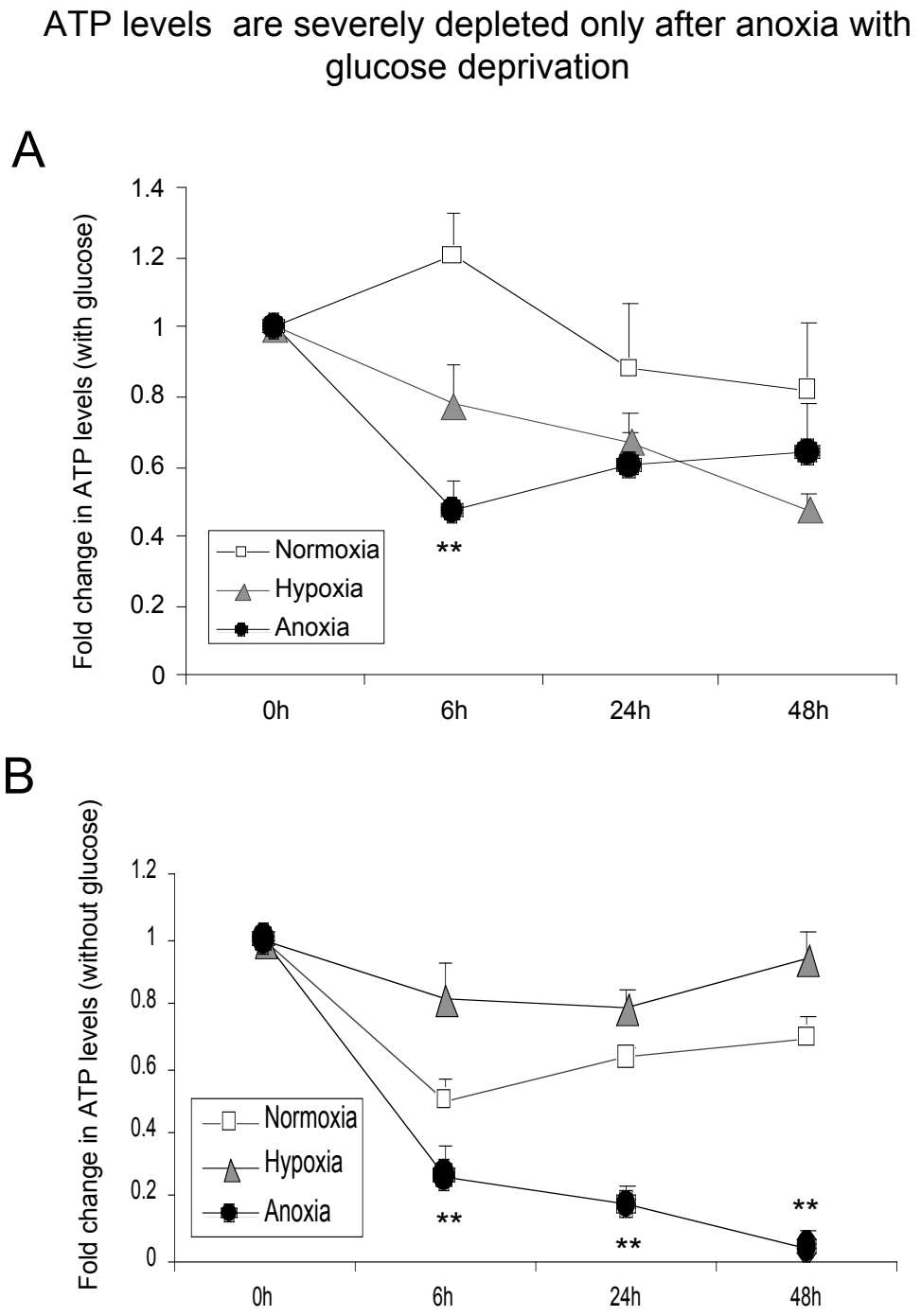

9 Curriculum Vitae

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Education

PhD Student, Zurich, Switzerland. 03.2003/present
Institute of Veterinary Physiology
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Title: "Hypoxia and the neuronal cytoskeleton: a role for cdk5/p35".

MSc in Clinical Neuroscience, London, UK. 09.1999/09.2000
Institute of Neurology, UCL
London, UK
Title of diploma thesis: "The effect of blocking β_1 integrins at the neuromuscular junction on re-innervation in adult rats."

BSc in Genetics, London, UK. 1996/1999
Queen Mary University, ULU
London, UK
BSc in Biology
Grade: 2:1
Title of thesis: "Molecular evolution of non-LTR retrotransposons in mosquitoes: Anopheles albimanus".

Greek Lyceum, Athens, Greece. 1992/1995
Grade: 17.8/20

A levels, Athens, Greece. 1994-1996
Subjects studied: English Literature, Physics, Chemistry, Biology

Work experience

Research assistant, Milan, Italy. 05.2001/03.2003
San Raffaele Hospital, Milan, Italy
Appointee, upon selection of a European Community research network grant. Worked on the genetic basis of migraine. The project: "Neuronal Ca^{2+} Channels in human disease".

Congresses

Antoniou X., Gassmann M.,Ogunshola O. Cdk5 regulates the neuronal hypoxic exposure through regulation of the Hif pathway. Society for Neuroscience, San Diego, USA, 2007.

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Publications

Schmid-Brunclik N., Antoniou X., Gassmann M., Ogunshola O. Astrocyte responses to injury: simultaneous cell death and proliferation. (Submitted to American Journal of Physiology-Regulatory, Integrative and Comparative Physiology).

Antoniou X., Gassmann M., Ogunshola O. cdk5 modulates Hif-1 in hypoxic neurons (submitted).

References

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10 Acknowledgements

I would like to thank my supervisor Dr. Ogunshola who taught me how to think in a scientific manner and was always there for me in the good and bad times. I would also like to express gratitude to my colleagues: Carole Bürgi Taboada, Abraham Al Ahmad and Stephan Keller for all their help in the lab and for putting up with me during periods of frustration. In addition, I would like to thank Professor Gassmann for giving me the opportunity to do my PhD thesis in his lab. Also I would like to express my thanks to Professor Sonderegger and Dr. Gesemann for careful reading of my PhD thesis. Last but not least I would like to thank Giovanni Camici for his patience but also for his scientific and psychological support throughout my PhD.